Edaphic and morphological factors affecting running buffalo clover (*Trifolium stoloniferum*) ecology

Research Thesis

Presented in partial fulfillment of the requirements for graduation

with research distinction in the undergraduate colleges of The Ohio State University

by

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Foreword

This thesis is presented in partial fulfillment of the requirements for graduation with research distinction in the undergraduate colleges of The Ohio State University. The research distinction program focuses on undergraduate original inquiry, as well as interdisciplinary interests. Investigation of the running buffalo clover, *Trifolium stoloniferum* Muhl. ex A.A. Eaton, falls within the scope of the undergraduate researcher because endangered species can persist across different conservation approaches. I chose to apply the agronomic perspective to the present issue of the species conservation, balancing other conservation programs in the species' research community.

The studies were selected in cooperation with the Ohio Department of Natural Resources, pursuant to permits CP 2017-5 and RP 2017-22. The Ohio Department of Natural Resources is the primary monitor and managerial agency responsible for running buffalo clover in the state, collating information from all landowners and site stewards to develop management recommendations, as well as move the species forward in a U.S. Fish and Wildlife Recovery Plan. The Ohio Department of Natural Resources is represented by the Chief Botanist based in Columbus. All running buffalo clover duties are carried out through the Division of Natural Areas and Preserves.

In relation to these regulatory duties, the Ohio Department of Natural Resources issued all necessary permits to approve research sampling from remnant populations. I applied and linked my research questions to the topics of edaphic relations, morphological characterization, and combined intervention-transplantation, with the hope that these insights and novel strategies might yield value to the overall species recovery plan.

Since November of 2016 I have been conducting research with the running buffalo clover, and with the approval of the Ohio Department of Natural Resources last May, I have been able to apply my research questions to wild Ohio specimens. Throughout this period I have become further interested in running buffalo clover and similar North American *Trifolium* species. At the beginning I had a limited knowledge of the species and the various strategies presented over the past 30 years of structured research on running buffalo clover. However, I have been able to achieve statistical and ecological results with which I am satisfied. I would like to thank my research advisor from the University, Dr. David Barker, the staff and volunteers of the Great Parks of Hamilton County, Marjorie Becus, Zuri Carter, and Jessica Spencer, the approval of the U.S. Fish and Wildlife Service, Jennifer Finfera, and the supporting Chief Botanist and Boch Hollow State Nature Preserve site manager at the Ohio Department of Natural Resources, Rick Gardener and Levi Miller, respectively. Their valuable insights and directions gave me the necessary background and support to complete essential steps in the research and write this thesis. Laus Deo.

Jonathan Omar Cole Kubesch April 2018

Abstract

Running buffalo clover, *Trifolium stoloniferum* Muhl. ex A.A. Eaton, (RBC) is an endangered North American true clover that has been a conservation enigma since its rediscovery in the early 1980s. Physiological and agronomic measures of the species haven't been a component of most research programs attempted in the past thirty-plus years.

My objective was to investigate edaphic and morphological factors affecting RBC ecology. I made it a sub-objective to use agronomy to address shortcomings in current ecological strategies. In many agro ecological contexts, similar Eurasian clovers can be propagated from several points in a life cycle; given this knowledge, similar propagation and plant evaluation should succeed for a North American *Trifolium*.

I studied the effects of pH on RBC growth, where in two experiments I examined RBC shoot and root growth (Chapter 2). In the first experiment, I studied growth under acidic and sulfurous regimes, where I found that RBC could persist in adverse conditions, but that white clover (*T. repens*) was a better competitor.

I examined the vegetative and reproductive growth of several RBC accessions in a greenhouse common environment where I found that plant material can be very similar within a population, but accessions varied in their ability to produce different forms of growth (Chapter 3).

In a greenhouse intervention and transplantation study I found that RBC can be propagated in the greenhouse from stolon tips (Chapter 4). These tips can yield up to 20 (average = 10) cloned plants for field plantings. Small transplants without a starter fertilizer planted in the fall appeared to be the most efficient transplants at this point in the process.

Overall RBC is a true clover with limited phenotypic plasticity. The species can modify habitat to suit its pH preferences. Regional accessions behave similarly to one another, and within accessions, some phenotypic plasticity exists, though not as much as previously hypothesized. RBC responds well to intervention strategies, and managing habitats according to phenotypes and addressing some pH conditions might improve success in the field.

To my loving family,

Thank you for encouraging my passion for the natural world

Acknowledgements

I'm indebted to a legion of individuals involved in the continuing existence of running buffalo clover. Without the interest and assistance of these individuals, this project wouldn't have succeeded. I want to first thank my research advisor, Dr. David Barker, for his supportive mentorship throughout this project, as well as for my initial introduction to the species. Many thanks to my thesis committee members, Dr. Emilie Regnier and Dr. Robert Klips, for their evaluation and suggestions.

I wish to thank the USDA GRIN network for providing accessions from Dr. Norman Taylor's running buffalo clover germplasm, the greenhouse staff at Kottman Hall for their greenhouse benches and patience, and the Ohio State University Department of Horticulture and Crop Science for allowing this undertaking.

Thanks to Rick Gardner and Levi Miller of the Ohio Department of Natural Resources, Jessica Spencer and Zuri Carter of the Great Parks of Hamilton County, and Jennifer Finfera of the U.S. Fish and Wildlife Service for permissions to collect Ohio material. Thanks for lending a few hours to measure plants on wet days in the woods. This includes Marjorie Becus, who al.so lent her expertise to backgrounding this thesis with previous accomplishments in the species' conservation

Thanks to Katie McNamara and Rafael Lupidi for their help in making initial measurements on the seemingly endless plant material. Thanks to Marko Jesenko for his insights into pollination ecology, and his introduction to pollination syndromes. Thanks to Dr. Matthew al.brecht and the Missouri Botanical Garden for access to Missouri and West Virginia seed stocks. Thanks to Dr. Reed Johnson and Chia Lin for the access and assistance in recording pollen observations.

Thanks to Dr. Michael Vincent for his taxonomic insights into the native clovers of Ohio and Kentucky. Thanks to Dr. Julian Campbell for his ecological and botanical descriptions of the Kentucky accessions used, as well as his call for the standardization of seed banking procedures and the common call to pool information about the species.

Vita

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Chapter 1: Introduction

Life History. Running buffalo clover (RBC), or *Trifolium stoloniferum*, is a federally endangered species, native to the Ohio River Valley, including states from Virginia to Missouri. It occurs in isolated patches in natural sites throughout southern Ohio in habitats often described as regions underlain with limestone or another similar material (Selbo et al. 2015). Currently 17 populations are known to state regulators, and of these populations a majority occur in Hamilton County, OH. These sites are generally mixed mesophytic forests, with dappled shade and intermediate disturbance regime maintaining the clover in the matrix (Selbo et al. 2015). The disturbance regime historically consisted of buffalo trampling paths to clear competing vegetation and fertilizing with manure. Hoof action assisted propagation by both spreading stolon material around and scarifying seed in the soil. . Where possible, sites are mowed 2-3 times annually to minimize weed competition. RBC lacks a rhizobia symbiont, and plants cannot fix nitrogen (Morris et al. 2006).

A perennial, the species colonizes areas with conspicuous stolons, often growing in large mats across the soil surface (Yatskievych 2006). The species is commonly confused with white clover, *Trifolium repens*, however it can be distinguished by using vegetative characters for identification in addition to the enlarged stipules and leaves of running buffalo clover (McKenzie et al.. 2018). Two similar species within the native range include the annual *Trifolium reflexum* and *Trifolium kentuckiense* (Chapel and Vincent 2013). Running buffalo clover flowers in May and June, setting seed in later June and early July; the inflorescences resemble those of white clover, except that running buffalo clover produces bracts on the flowering stalk similar to those expressed in red clover, *Trifolium pratense* (Yatskievych 2006).

History and current state in the wild. Within Ohio, running buffalo clover was sufficiently common that numerous botanical collectors mounted a plethora of specimens; as a consequence, a historical sites populate the Appalachian Hocking Hills and Bluegrass Cincinnati regions (Selbo et al 2015; Marjorie Becus 2017, pers. comm). The species was presumed extinct from the 1940s until the 1980s, when the rediscovery of several sites in West Virginia, Kentucky, and Ohio. The first Ohio sites were located in Hamilton County, and the botanist Allison Cusick collected voucher specimens from these plants which remain the most recent state specimens publicly available for the species. Habitat loss, genetic bottlenecking, and disease pressures have all been suggested mechanisms of running buffalo clover decline (Selbo et al. 2015). This preservation of sites led to a period of in-situ conservation, where historical populations were conserved by managing sites rather than propagating plant material in the greenhouse.

Loss of mesic savanna and open habitats has contributed to RBC becoming a federal endangered species. Previous investigators have documented natural populations after the species was rediscovered in the mid-1980s (Yatskievych 2006). Ohio State University students and faculty studied much of this federally endangered species. Twenty years before the present study, graduate students in Ohio State University's former Department of Plant Biology as well as the School of the Environment and Natural Resources studied the soil characteristics of RBC areas, as well as the fecundity of natural populations (Hattenbach 1996, Franklin 1998). Ohio State alumni worked on the U.S. Fish and Wildlife Recovery Plan for the species (Selbo 2007). By conducting ex-situ research, propagating plant material outside of historical sites, RBC continues to exist in research laboratory populations (Sparks and Barker 2013).

Previous investigators have documented natural populations by happenstance encounters since the species was rediscovered; in a famous anecdote, the first Missouri plant material was discovered in a neglected topsoil pile on the property of state botanist Dr. George Yatskievych (Yatskievych 2006). Rediscovery across the eastern historical range resulted in several intervention conservation efforts at this time, several of which solely focused on producing field transplants or growing plants for seed. To these ends, a Missouri strategy of ex-situ propagation followed by in-situ transplantation and an ex-situ seed collecting Kentucky strategy developed across several research groups in both states. In contrast, other than documentation and some seed collections, populations in West Virginia and Indiana were managed as part of their protected communities and ecosystems (Selbo et al 2015; Dr. Matthew al.brecht 2018, pers. comm).

Following Dr. Yatskievych's fortuitous discovery, the Missouri Department of Conservation [MDC], in conjunction with the Missouri Botanical Garden [MOBOT], staged several reintroductions with limited success. These initial plantings consisted of material propagated from Ohio, West Virginian, and Kentucky plants. The plantings persisted as long as 8 years under annual monitoring, with more than 50% disappearing in the first three years. (Smith 1998). These efforts were abandoned upon the rediscovery of nearby endemic populations (Smith 1998).

More directly, Dr. Norman Taylor at the University of Kentucky [UK] collected and maintained numerous *Trifolium* species from around the world, including collections from both annual buffalo clover (*T. reflexum*) and running buffalo clover. Following his death in 2012, these accessions joined the USDA-GRIN. It must be noted that MOBOT collected and still collects seed from wild populations; however, unlike Taylor, MOBOT didn't maintain large seed-producing populations in captivity; according to historical propagation notes, greenhouse plant material was maintained for vegetative cloning rather than seed increases; even presently

MOBOT banks seed collected from wild source populations (Dr. Matthew al.brecht 2018, pers. comm).

Two research institutions in the state of Ohio examined Ohio and Kentucky populations since the species rediscovery: Miami University (Oxford, OH) and The Ohio State University (Columbus, OH). Under the direction of herbarium curator Dr. Michael Vincent Miami researchers have investigated the morphological, genetic, taxonomic components of running buffalo clover populations in the wild. Ohio State University students and faculty studied this federally endangered species in much the same way.

Twenty years before the present study, graduate students in the former Department of Plant Biology as well as the School of the Environment and Natural Resources studied the soil characteristics of running buffalo clover areas, as well as the fecundity of natural populations (Hattenbach 1996, Franklin 1998). These masters' theses investigated remnant populations, rather than building living sample inventories. Hattenbach (1996) documented the soil conditions around the population. RBC grew in conditions slightly different from nearby habitat. In this survey of the population sites, Hattenbach observed that the plants grew in locally enriched pockets within a site. RBC germination improved with sulfuric acid degradation of the seed coat (Hattenbach, 1996).

Franklin (1998) studied seed set and pollination of RBC field populations. Franklin confirmed the plants were capable of self-fertilization, but required pollinators to 'trip' the keel petal and release pollen. Franklin did not investigate seedling recruitment, but found that seed production was limited, especially in unfavorable years.

Hattenbach suggested future avenues for research during the species' last return from obscurity (Hattenbach, 1996). A revived interest in the Department of Horticulture and Crop Science lead to the development of a sizable greenhouse research collection comprised of populations from Ohio as well as seed accessions from West Virginia, Kentucky, and Missouri.

Despite some ex-situ research projects suggested by Hattenbach (1996) coming to fruition over the past two decades—growth under different light regimes, seed viability following passage through a ruminant digestive tract, and vegetative vs. sexual reproduction—running buffalo clover languishes in monitoring projects in the wild (Perkins, 2015). Outside of Ohio, UK disassembled its clover collection for eventual donation to the USDA. The shift by MOBOT from producing plant material to occasionally collecting wild seed has limited acquisition about species propagation; even USDA-GRIN, which presently maintains Taylor's accessions, only carries out seed increases rather than research evaluation. Though Margo Price's popular southern rock band Buffalo Clover briefly brought attention to native clovers, outreach to the wider public remains limited to the Ohio Department of Natural Resources [ODNR] and MOBOT. Access to seed remains an academic privilege not presently explored commercially, and public awareness is low (Barker and Sparks 2013; Buffalo Clover ft. Margo Price; Rick Gardner 2017 pers. comm). Species research remains fragmented amongst disparate groups, with Ohio experts working primarily on managing site community ecology (McKenzie et al. 2018; Paul McKenzie pers. comm.).

Justifications. In the past five years, Dr. David Barker, Department of Horticulture and Crop Science, worked with running buffalo clover to develop applications that might encourage running buffalo clover's preservation (Sparks and Barker 2014, Barker and Sparks 2013). By advocating for further research, Dr. Barker ensures that running buffalo clover continues to exist in research laboratory populations (Barker and Sparks 2013). Various conservationists, though united by the species, remain divided in their research strategies. Through the following collation of research and conservation efforts, the author addresses the diffuse nature of previous research groups, and to connect their work without harking back to some social construction of pristine nature. This thesis seeks to explore pressing conservation and biological questions in the species in order to support conservation initiatives for both the ODNR and the U.S. Fish and Wildlife Service (Gardner et al... March 27, 2017, pers. comm.). Working with regulatory and conservation agencies, the author complements field efforts with basic and translational programmatic research. Additionally, unifying the Missouri and Kentucky historical conservation strategies could improve holistic efforts both in-situ and ex-situ.

Statement of Objectives

I investigated edaphic and morphological factors affecting RBC ecology. Using material from several sources I was able to propagate a sizable collection for my studies in both the greenhouse and the field. A sub objective was to integrate agronomy applications to the ecological problems facing RBC; ideally this strategy would circumvent shortcomings from previous ecological approaches to species research.

Edaphic factors: the different soil conditions present across the three major regions and within these regions suggest that RBC may be phenotypically plastic to tolerate such conditions. As nutrient availability is tied to pH for many key elements, pH was investigated. Given previous literature suggests RBC exists in alkaline growing conditions, then the species likely grow best in those conditions like domesticated alfalfa (*Medicago sativa*)(Hattenbach 1996). Following upon original field studies, I explored performance under varying pH regimes (Hattenbach 1996). Given the potential fitness consequences of specific edaphic regimes, such determinations may aid future searches and screens for yet discovered populations. In the pH study I observed agronomic performance under different pH regimes to ascertain pH optima as well as comparative growth with common forage legume species. Examining this growth will help determine the ideal soil conditions for finding remnant populations, and developing optimal transplantation strategies. This could al.so support experimental population site selection or management.

Morphological factors: given that running buffalo clover is a relatively wild species, populations may differ from one another across a fragmented range. Ozark MO material won't produce the same agronomic or edaphic responses as the Bluegrass or Appalachian material might. If the genetic data suggests homogeneous populations that differ between one another, then genetic diversity exists between, rather than within, populations.

Running buffalo clover ecology: in a postlapsian climate, interventions into the populations of species are necessary, perhaps even crucial. I collected vegetative stolons from wild populations to rear specimens for field transplants. Developing optimal protocols for transplantation would assist present population relocations as well as identify successful strategies for setting up experimental field populations. If field transplants can survive an establishment period—overwintering and one stolon growth cycle—then greenhouse intervention and transplantations might overcome the precarious boom-bust dynamics presently observed. Should cautions be taken to represent the sampled population and compensate for differential propagative success, plantings can minimize selective biases away from the natural population fitness.

Chapter 2: The Effect of soil pH on the growth of running buffalo clover

*Note: this chapter is revised from a manuscript in preparation for Plant and Soil

Introduction

RBC is a federally endangered species, native to the Ohio Valley. It occurs in isolated patches in natural sites throughout southern Ohio. Its habitat is often described as regions underlain with limestone having a pH 6.8-7.2 (Selbo et al. 2015). However, soil descriptions of naturally occurring populations vary across the species range (Gardner et al.. 2017, pers. comm; Hattenbach, 1998). Working through species literature, edaphic studies investigating such habitat pH indicators remain rare in the time since Hattenbach (Becus 2017, pers. comm.; Hattenbach 1996). Complete soil testing revealed that the species occupies nutrient-rich pockets of soil, but not adjacent, poorer ground (Hattenbach 1996). As expected, the species presence across a wide pH range allows it to persist in West Virginian and Ohioan Appalachia as well as the in the Bluegrass region of southwestern Ohio and northern Kentucky. Within regions, accessions may respond similarly, but pH optima may vary among regions.

Loss of savanna and forest clearing habitat has resulted in RBC becoming a federal endangered species. Presumed extinct around 1940, researchers rediscovered small populations in the mid-1980s along the Ohio River corridor (USFWS 2007, USFWS 2011). Previous research has investigated the forage potential of RBC, however, it has failed to outperform other legume species, white clover (*T. repens*) (Barker and Sparks 2014).

Since the primary threat to running buffalo clover is loss or modification of habitat (Selbo et al, 2015), one option for restoration of this species is identification of suitable edaphic areas for re-introduction. The soil pH requirements for such re-introductions are uncertain. The surveying of habitat without RBC may find different soil conditions than within RBC-populated areas. Furthermore the documented differences in pH range between the Bluegrass and Appalachian regions may elicit varying responses between these distinct regions (Hattenbach, 1996; Crawford et al.. 1998).

Objectives

I sought to determine the effects of pH on different RBC accessions, as well as compare how RBC compares to agricultural legumes. Determining pH optima for RBC growth would, with the additional of other ecological indicators, screen the range for potential remnant populations or selecting ideal planting sites (Burkhart et al. 2013; Morris et al. 2002; Chapin et al. 2011). Habitat specificity produces management implications, and with pH determining nutrient availability, pH optima may al.so correlate with site suitability manipulations (Hattenbach 1996; Brady and Weil 2010; Morris et al.. 2002; Chapin et al.. 2011). Previous work identified that RBC occurs in pockets of enriched soil within the range and that pH within these patches can differ from the surrounding site (Hattenbach 1996). No studies so far have evaluated how well the species grow using any measures of growth, such as shoot or root mass.

Methods

This study comprised two experiments conducted in the Kottman Greenhouse, Ohio State University, Columbus OH. Experiment 1 ran from 16 December 2016 until 26 March 2017, and Experiment 2 ran from 9 May 2017 until 28 November 2017. In both experiments, the experimental unit was two-four plants grown in a 1.5 L pot (10 x 10 cm, 15 cm height). Both experiments used USDA Kentucky RBC accessions: seed lots several generations removed from source wild populations. Vermiculite media was used in both experiments to facilitate harvesting of roots. In addition to media treatments described below, plants were supplied with Peters 200 ppm nutrient solution approximately weekly during the experiments.

Experiment 1

Exp. 1 comprised four plant treatments [three accessions of RBC, and white clover var. 'Jumbo'] in a factorial arrangement with two pH (5.0 and 6.0) and two $FeSO_4$ levels (0 and 1000 mg S/L). The experimental design was a 4 x 2 x 2 factorial treatment structure and a randomized complete block arrangement of four replications (64 pots in total). The RBC plant material came from three USDA accessions (PI numbers: 641566, 22231, and 31415). The pH 5.0 + 0 mg S/L treatment was untreated tap water, acidified with H_2SO_4. The pH 6.0 + 0 mg S/L treatment was untreated tap water. The pH 5.0 + 1000 mg S/L treatment was 0.3 M $FeSO_4$ solution. The pH 6.0 + 1000 mg S/L treatment was 0.3 M $FeSO_4$ solution, adjusted with NaOH. The $FeSO_4$ solutions were used since acid soils in Ohio are frequently associated with elevated levels of Fe and S.

Exp. 1 comprised a 5-wk establishment phase (16 December 2016 to 20 January 2017) and a 9-wk treatment/measurement phase (20 January to 28 March 2017). During the establishment phase, plants were established directly within the pots from stolon tips removed from mature nursery plants as described by Sparks and Barker (2013a). Plants were watered approximately daily and did not receive pH nor S treatment. During the treatment/measurement phase plants received three to four applications per week of the respective pH-S solutions in lieu of watering. Plant diameter, and stolon number and length, plant vigor (1=poor, 5=excellent), active growing points, and leaves per plant was measured at 6 and 9-wk of the pH and S treatment period. Plant diameter was calculated as the average of the longest and smallest dimension of individual plants. Root media pH was measured on 22 February and 26 March, 2017. On 26 March 2017, the above-ground vegetation was harvested, and vermiculite was washed from roots. Dry weight was measured after 40 hr at 60^oC.

Experiment 2

Exp. 2 comprised eight plant treatments [five accessions of RBC, and three legume species: alfalfa (*Medicago sativa* L. cv ‘55VR08’), birdsfoot trefoil (*Lotus corniculatus* L. cv ‘Viking’), and white clover cv ‘Jumbo’] and three levels of ‘soil’ modification (acidified, de-acidified, and untreated). The experimental design was an 8 x 3 factorial treatment structure and a randomized complete block arrangement of four replications (96 pots in total). The RBC plant material came from three USDA accessions [PI numbers: 641565, 641566, 667999 (a seed increase from 22231, that was used in Exp. 1), 631732, and 31415].

Exp. 2 comprised an 18-wk establishment phase (9 May to 29 August 2017) and a 10-wk treatment/measurement phase (20 September to 28 November 2017). Seed of RBC, white clover, alfalfa, and birdsfoot trefoil was planted in excess, and thinned to two plants per pot for RBC and white clover, and four plants per pot for alfalfa and birdsfoot trefoil. Given the limited seed supply for RBC, scarification and germination protocols were used as described by Sustar (2017). During the establishment phase, the acidification treatment used an approximately equal mixture of elemental sulfur and gypsum (‘Fast Acting Sulfur’, Encap LLC, WI) incorporated in the vermiculite prior to planting at a rate of 250 g/m^2 (the recommended rate for individual small plants; 1 teaspoon per plant, 2.5 g/pot). The product comprised Ca 11%, S 49%, and 6.4% of inert ingredients. The de-acidification treatment used lime (‘Fast Acting Lime’, Encap LLC, WI) incorporated in the vermiculite prior to planting at a rate of 250 g/m^2 (the recommended rate for individual small plants; 1 teaspoon per plant, 2.5 g/pot). To partially offset the addition of S in the acidification treatment, gypsum ($CaSO_4.2H_2O$) was included at the same rate. The product comprised Ca 11%, S 39%, and 5.5% of inert ingredients, and the mixture comprised approximately Ca 31%, S 9%. Since the media was vermiculite and the plants were watered to excess on occasion, the acidification and de-acidification applications were repeated approximately monthly, with a surface applications at the same rate.

The measurement period for Exp. 2 was 20 September-28 November 2017. Media pH measurement (on 15 September 2017) showed the acidification and de-acidification treatments did not have much difference in pH, and plants were subsequently irrigated with different pH solutions. Acidic treatments received a pH 4 solution generated from H_2SO_4, whereas the basic treatment received a pH 8 solution generated from NaOH. The untreated (control) treatment continued to receive tap water and the nutrient solution application schedule remained the same. These pH treatments were applied two-three times/week during 20 September to 16 November, 2017.

Plant mass (5 cm above ground level) was measured three times during Exp. 2, 29 August, 10 October, and 28 November, 2017. All harvested material was weighed after 48-hour drying at 60^{o}C. Roots were washed from the vermiculite on 28 November, and dry weight determined as for shoots. Media pH was measured on 21 November.

Statistical analysis

Exp. 1. All data collected throughout the experiment was recorded using Microsoft Excel, and then analyzed statistically using SAS. The experimental unit was the individual pots in a 4 x 2 x 2 treatment structure: 4 plant accessions, a presence or absence of both sulfuric acid treatment and/or $FeSO_4$ treatment. Alpha level was set to p=0.05. The main effect for plant had 3 degrees of freedom and this was partitioned into an effect due to species (2 species – 1 = 1 degree of freedom), and accession-within-RBC (3 accessions – 1 = 2 degrees of freedom). Comparisons were made among the white clover and 3 RBC accessions used.

Exp 2. The experimental unit was an individual pot in an 8 x 3 treatment structure: 8 plant accessions, and three pH conditions. All data collected throughout the experiment went into Microsoft Excel spreadsheets. Analyses were conducted in R (version 3.4.3) (R Core Team 2013). Alpha level was set to p=0.05. The main effect for plant had 7 degrees of freedom and this was partitioned into an effect due to species (4 species – 1 = 3 degrees of freedom), and Accession-within-RBC (4 accessions – 1 = 3 degrees of freedom). Comparisons were made at three levels: among the 3 legumes and 5 RBC accessions; among the 3 legumes and an average of the RBC accessions; and among the RBC accessions.

Results

Experiment 1

When the nutrient solution treatments began on 20 January 2017, RBC plants averaged 0.549 g of shoot, and 0.481 g of root, whereas white clover averaged 0.0825 g of shoot, and 0.0694 g of root. After 9 wk of treatment, the SO_4 main effect was statistically significant on all shoot metrics, though the pH main effect was not (Table 2.1). Interactions between species, pH, and SO_4 occurred for leaf count, plant diameter, and stolon length (Table 2.1, Figures 2.1-4). all metrics had significant SO_4 effects (Table 2.1, Figure 2.1). A species x pH x SO_4 interaction occurred for several measurements (Table 2.1, Figures 2.3-4, 2.6).

Plant and $FeSO_4$ main effects were significant on shoot dry weight (Table 2.2, Figure 2.5), however the species effect was not significant. Interactions of accession x SO_4, as well as species x pH x SO_4, were al.so significant (Table 2.2). Root dry weight had significant species, accession, and $FeSO_4$ effects, though a species by pH x SO_4 interaction was al.so significant (Table 2.2, Figure 2.6). Root and shoot mass varied among RBC accessions, however white clover produced more root mass compared to RBC (Figure 2.6). Treatment effects on root-to-shoot ratio were not significant. In this experiment, running buffalo clover appeared to acidify their inert vermiculite medium.

Experiment 2

Second cutting 10 October 2017. Significant species, treatment, and species-treatment effects occurred for shoot growth at the second cutting. The running buffalo clover accessions produced the least shoot dry mass of the four species; however, the 565 accession was the most productive of the five RBC accessions. Initially the acid and base treatments had reduced growth relative to the control treatment, but eventually the basic and control treatments had comparable growth.

Third cutting 28 November 2017. At the third cutting, only the species effect was significant, with all three comparison legumes out-producing running buffalo clover. Alfalfa was the most productive species in all parameters, and RBC had the least productive root and shoot mass (Table 2.3). Alfalfa and white clover produced similar shoot mass, whereas white clover and running buffalo clover produced similar root-shoot ratio.

For all growth parameters, no significant differences existed amongst the RBC accessions in the third cutting. Each accession behaved similarly to one another. Alfalfa was consistently one of the most productive species, in root and shoot production. Even in the ratio of root-shoot, the species produced large values (Table 2.3).

The differences among substrate pH responses confirmed the existing notions pH optima of the comparison legumes. Despite receiving the same acidifying or basifying solutions, the species modified substrate pH differently. The basic and neutral treatments weren't significantly different from one another, and the acidic treatment was significantly different from both (Table 1.4).

Discussion

pH treatment

Media pH had significant effects on RBC growth and morphology in both Exp.1 and Exp. 2. These effects might be due to direct effects of pH, as well as effects due to nutrient availability (which can vary with pH). Running buffalo clover appeared to grow best in basic and neutral soil conditions; however the species appeared to acidify its vermiculite medium. Running buffalo clover compares poorly with agricultural legume species.

The observed results in the greenhouse might explain growth of RBC across a broad range of soil pH in its natural environment. Given that the documented field populations had similar pH values compared to those observed in Exp. 2, nutrient availability may not be determined by edaphic controls, but rather by deposition by animals (Hattenbach 1996; Morris et al... 2002). Given field populations don't receive controlled fertilizer inputs, and that field pH differences weren't observed, running buffalo clover isn't narrowly specific to local pH conditions, nor does the species significantly alter the site pH (Barak et al... 1997; Bloom et al... 2005).

Running buffalo clover appeared to acidify their inert vermiculite medium (Exp. 1 & 2). Acidification of soil by plants is a result expected from calcareous substrate plants; interacting with the rhizosphere, the roots exchange acidic protons for nutrients, and thus in an inert medium the roots will gradually acidify the soil solution. This result from Bluegrass region USDA accessions supported the original botanical conclusions regarding species distribution: namely, that the clover can physiologically function on calcareous soil. While true in the Bluegrass region, this acidification hypothesis may not fully explain sites in acidic Appalachia, such as Boch Hollow State Nature Preserve (Selbo et al 2015).

SO_4 treatment

RBC and white clover responded negatively to the SO_4 treatment. RBC reacted more negatively than white clover, with a greater decline in growth. Both species preferred the control conditions with higher pH conditions and the absence of SO_4.

Plant species

The differences among species may relate more to the morphological structure of the legumes. The stolon-generating clover species exhibited similar root-shoot ratios to one another as did the crown-generating legumes. Further comparisons amongst taxa with morphologically similar structures may need to be used. The distribution of roots differs in that a crown may center under the main shoots whereas the stolons may spread the root system across a diffuse network.

Whereas domesticated legumes such as alfalfa, birdsfoot trefoil, or white clover might be bred for narrow or wide pH growth optima, the natural populations of RBC haven't been selective or bred for field performance. This might explain the reduced root and shoot growth relative to the comparison legumes.

The significant differences among accessions for shoot growth (Table 2.1) suggests minor morphological differences between running buffalo clover accessions; however, previous genetic investigations hint at regional differences with relatively homogeneous populations (Crawford et al.. 1998). The differential success of wild populations, and the boom-bust cycles observed in ecology of the species may be tied to minute differences in edaphic conditions at a fine-scale.

The putatively underperforming RBC may owe part of its modest vegetative colonization to this limited shoot growth.

Reduced plant size tends to be associated with limited stolon colonization. In a species where stolons serve as the primary means of reproduction, shoot growth may correlate to reproductive fitness at both the organismal and population level. In the white clover model system, and in running buffalo clover population genetics, stolons determine the fate of populations more so than seed banking in the soil (Chapman 1983, Chapman and Anderson 1987 I and II; Crawford et al.. 1998). More vigorous, hardy populations might perform better than the accessions currently available, or investigations may prove 31415 to be an outlier of the species (Figures 2.1 and 2.5).

Morphological characterization might serve as a heuristic for genetic analysis to determine inter and intra-population responses to similar adverse conditions. Further investigations might include unique RBC populations from elsewhere in the species' range (Crawford 1998). However, some specific accessions might be better suited for future commercial applications. Further comparative studies would better evaluate the diversity of potential wild varietal candidates (Figures 1.1 and 1.5). The genetic diversity of RBC offers a suite of potential sources for reclamation, and additional data suggests that accession 31415 represents the most useful agronomical accession of those used in this study. Given the superior agronomical performance of white clover, running buffalo clover appears an ineffective candidate for mine reclamation purposes (Tables 2.1 and 2.2).

Despite the present conclusions, future studies may find running buffalo clover accessions that may encourage mine reclamation applications, and conservation strategies may experiment with mixed establishment strategies, such as using a grass nurse crop to hold soil during the clover's establishment. Field studies of establishment dynamics in similar conditions should follow from greenhouse evaluation of accession; ideally monitoring programs similar to current conservation measures would improve such field studies (Perkins 2015).

Understanding the stolon promotion from disturbance regimes (eg regular shoot cuttings) may offer fitness insights into species' edaphic interactions. Acidification of soil by plants is a result expected from calcareous substrate plants (Hattenbach 1996; Brady and Weil 2010). Interacting with the rhizosphere, the roots exchange acidic protons for nutrients, and thus in an inert medium the roots will gradually acidify the soil solution.

Future investigations of the species might explore the physiological and chemical mechanisms for running buffalo clover to acidify its media, as well as additional comparisons amongst running buffalo clover populations. Measurements of root density and comparisons with morphologically similar taxa may al.so tease apart subtle differences that weren't apparent in the present study. Running buffalo clover appeared to acidify their inert vermiculite medium (Experiment 1). Acidification of soil by plants is a result expected from calcareous substrate plants; interacting with the rhizosphere, the roots exchange acidic protons for nutrients, and thus in an inert medium the roots will gradually acidify the soil solution. This result from Bluegrass region USDA accessions supported the original botanical conclusions regarding species distribution: namely, that the clover can physiologically function on calcareous soil. While true in the Bluegrass region, this acidification hypothesis may not fully explain sites in acidic Appalachia, such as Boch Hollow State Nature Preserve (Selbo et al 2015).

Running buffalo clover appears to grow best in basic and neutral soil conditions; however the species appeared to acidify its vermiculite medium. Running buffalo clover compares poorly with agricultural legume species.

Table 2.1 Exp. 1. Significant effects for plant traits measured 26 March 2017

Shoot Variable	Treatment Effect or Interaction	Pr>F
Vigor	Species (S)	0.0129
	Variety (V)	0.0329
	Sulfate (SO_4^{2-})	0.0284
Active Growing Points	S	<0.0001
	V	0.0375
	SO_4^{2-}	0.0003
	Species x SO_4^{2-}	0.0344
Leaves	SO_4^{2-}	<0.0001
	S x A (A) x Sulfate	0.0006
Plant Diameter	Sulfate	0.0002
	Species x A x Sulfate	0.0266
Stolon Length	Sulfate	0.0009
	Species by Acid by Sulfate	0.0114
Stolon number	Sulfate	0.0004

Table 2.2 Experiment 1. Significant effects for plant traits measured on 26 March 2017

Dry Weight	Treatment Effect or Interaction	Pr>F
Shoot	Variety	<0.0001
	Sulfate	<0.0001
	Variety by Sulfate	0.0440
	Species by Acid by Sulfate	0.0003
Root	Species	0.0378
	Variety	<0.0001
	Sulfate	<0.0001
	Species by Acid by Sulfate	0.0031

Table 2.3. Experiment 2. Plant treatment effects at the end of the experiment 28 November 2017. Note: Fisher's protected LSD's for columns.

Species and Accessions	Final Cut Shoot (g DM/pot)	Root (g DM/pot)	Root-shoot ratio
Alfalfa	6.46a	11.99a	2.28a
Birdsfoot trefoil	3.54b	8.04b	2.22a
White clover	6.31a	5.10c	0.82b
RBC #231	2.61b	1.45d	0.58b
RBC #415	2.26b	1.30d	0.56b
RBC #565	3.19b	1.53d	0.48b
RBC #566	2.77b	1.77d	0.66b
RBC #732	2.54b	1.65d	0.64b

Table 2.4. Exp. 2. Media pH values for plant treatment 21 November 2017.

Species and Accessions	Acidic media	Basic media	Neutral media
alfalfa	6.44	6.89	7.00
birdsfoot trefoil	6.67	6.87	6.71
white clover	6.15	6.68	6.60
RBC #231	6.33	6.54	6.43
RBC clover #415	5.81	7.10	6.72
RBC #565	6.10	6.70	6.56
RBC #566	5.84	7.0575	6.60
RBC #732	6.0975	6.725	6.69

Figure 2.1 Experiment 1. Species/Accession effect for active growing points per plant (p=0.0329).

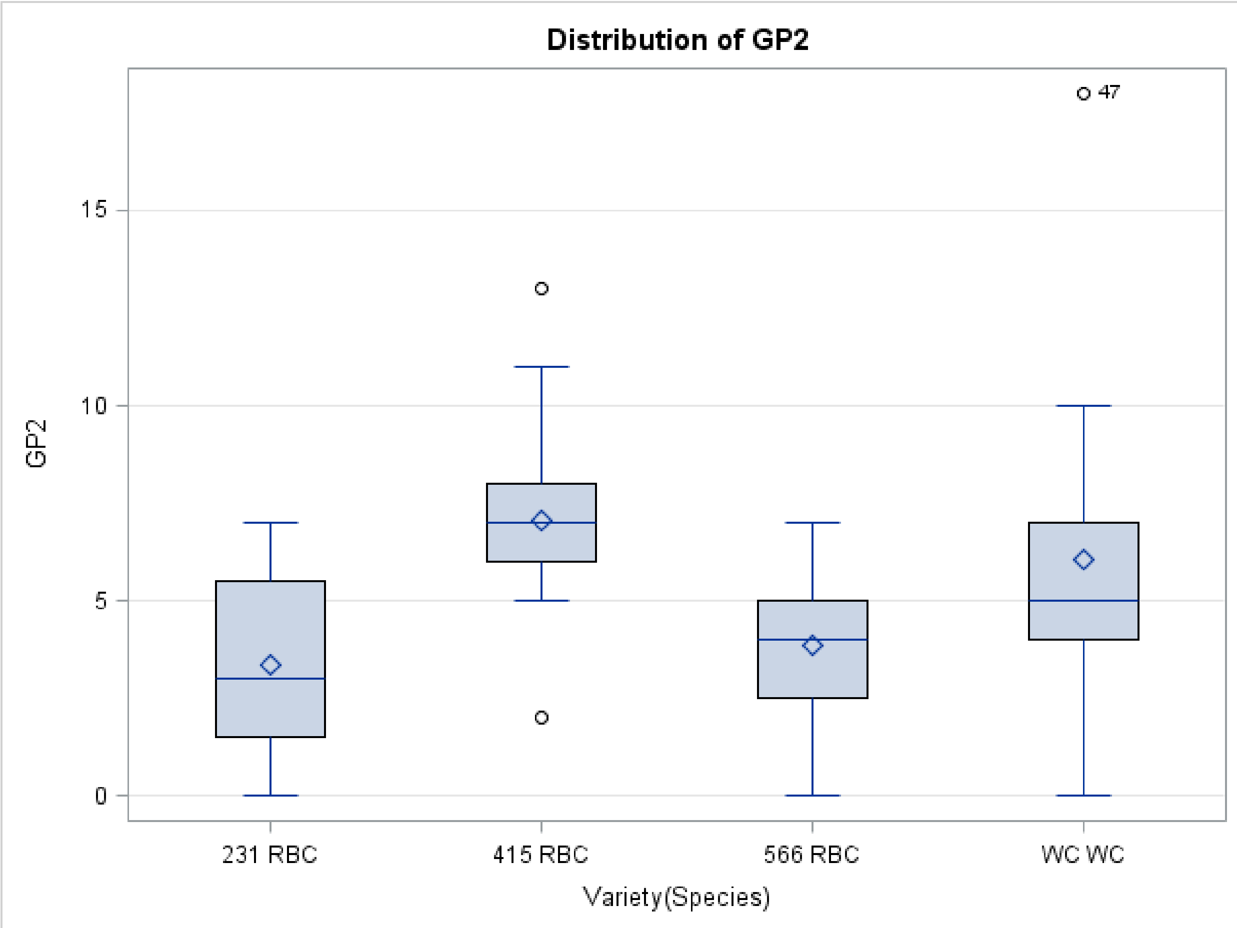


Figure 2.2 Experiment 1. Active growing points species by sulfate interaction (p=0.0244)

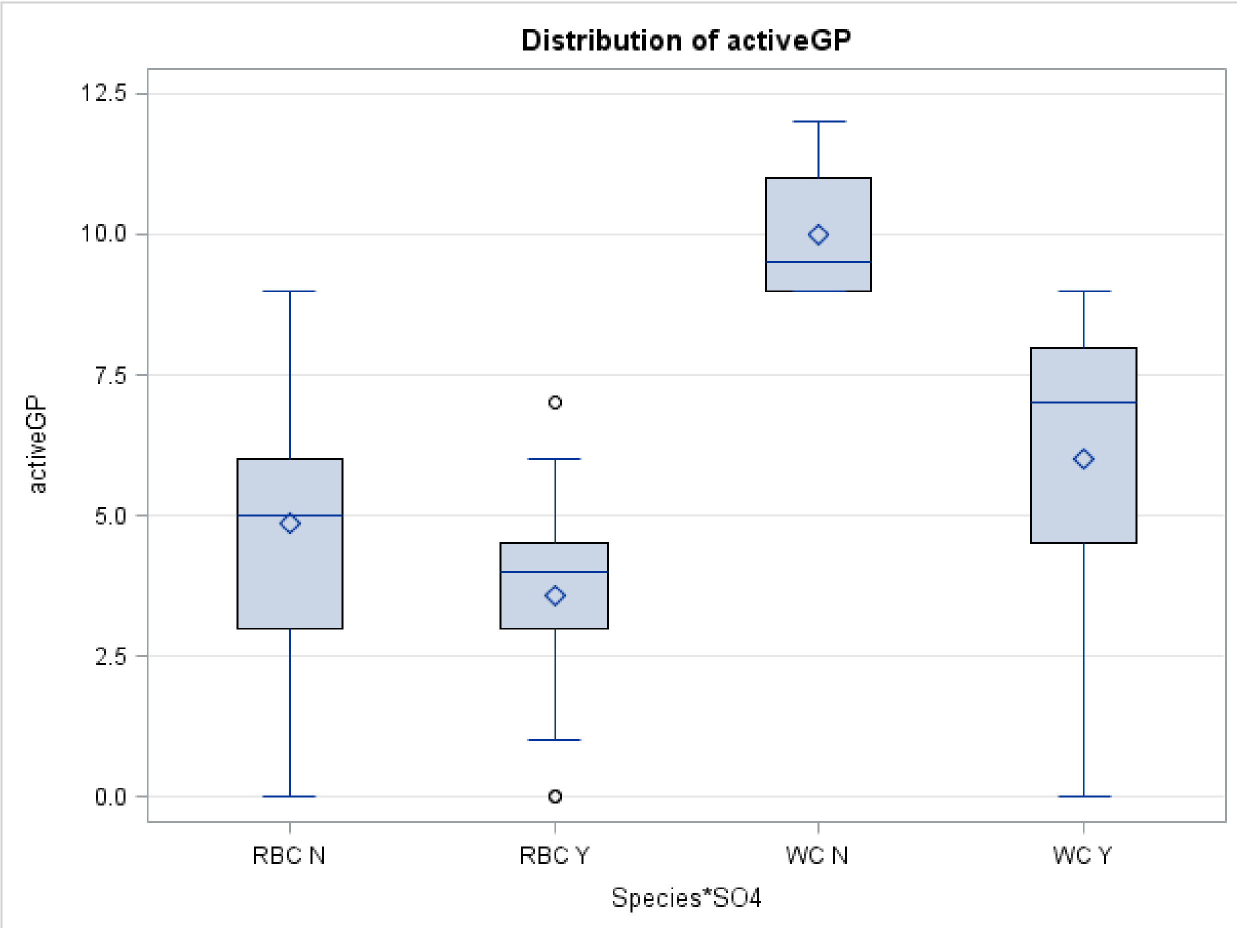


Figure 2.3. Experiment 1. Leaf count species by acid by sulfate interaction (p=0.0006)

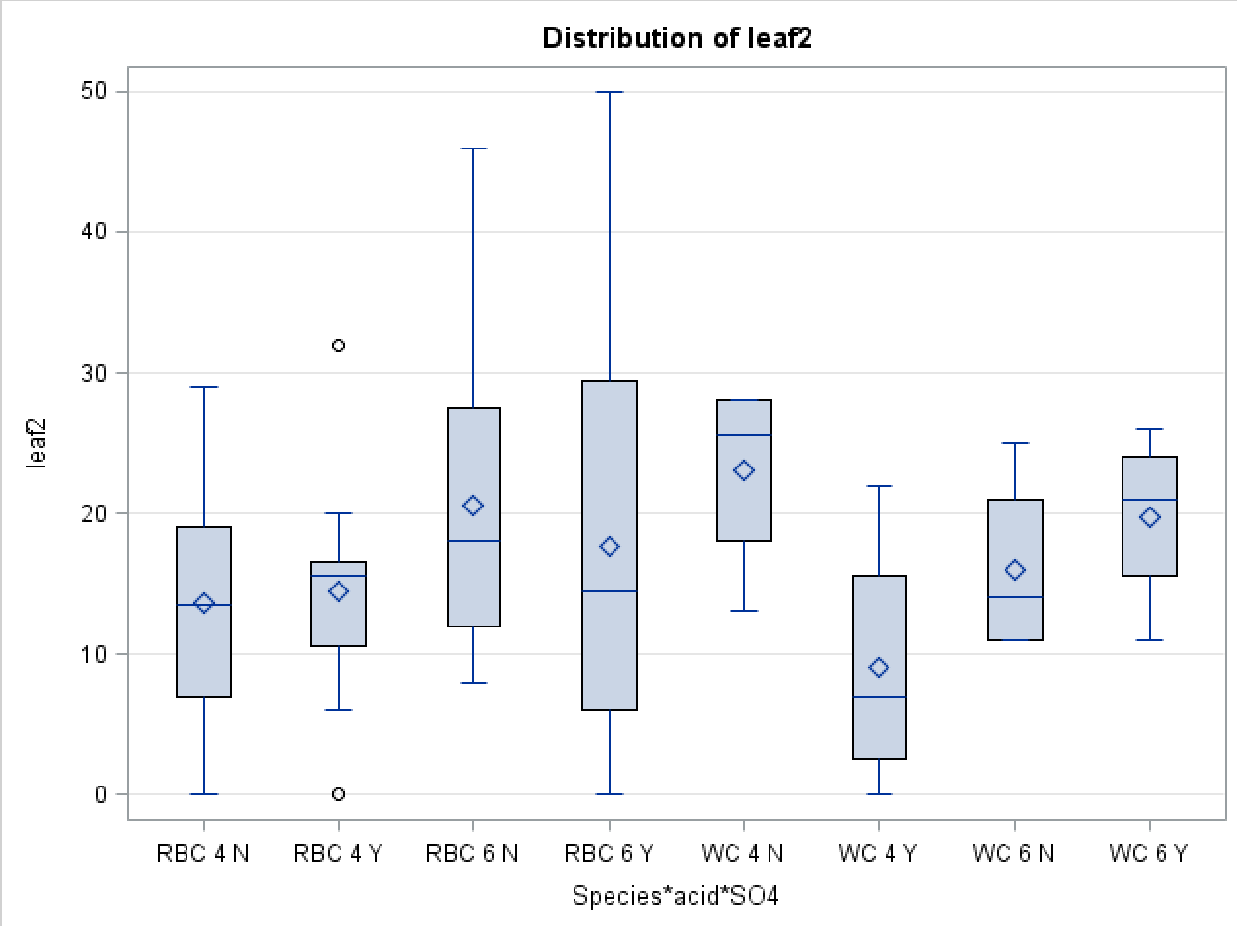


Figure 2.4. Experiment 1. Diameter species by acid by sulfate interaction (p=0.0266)

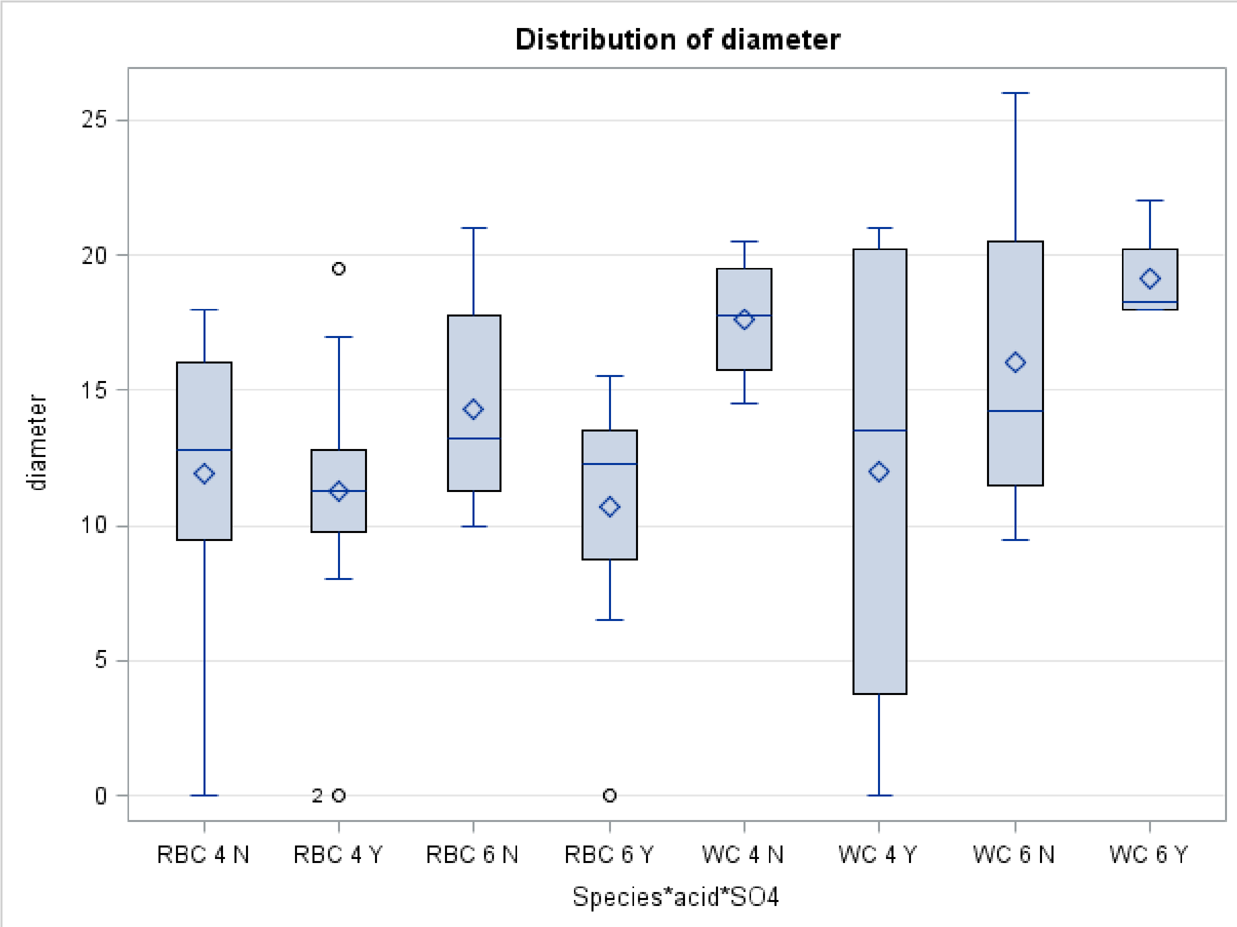


Figure 2.5. Experiment 1. Shoot dry weight variety by sulfate interaction

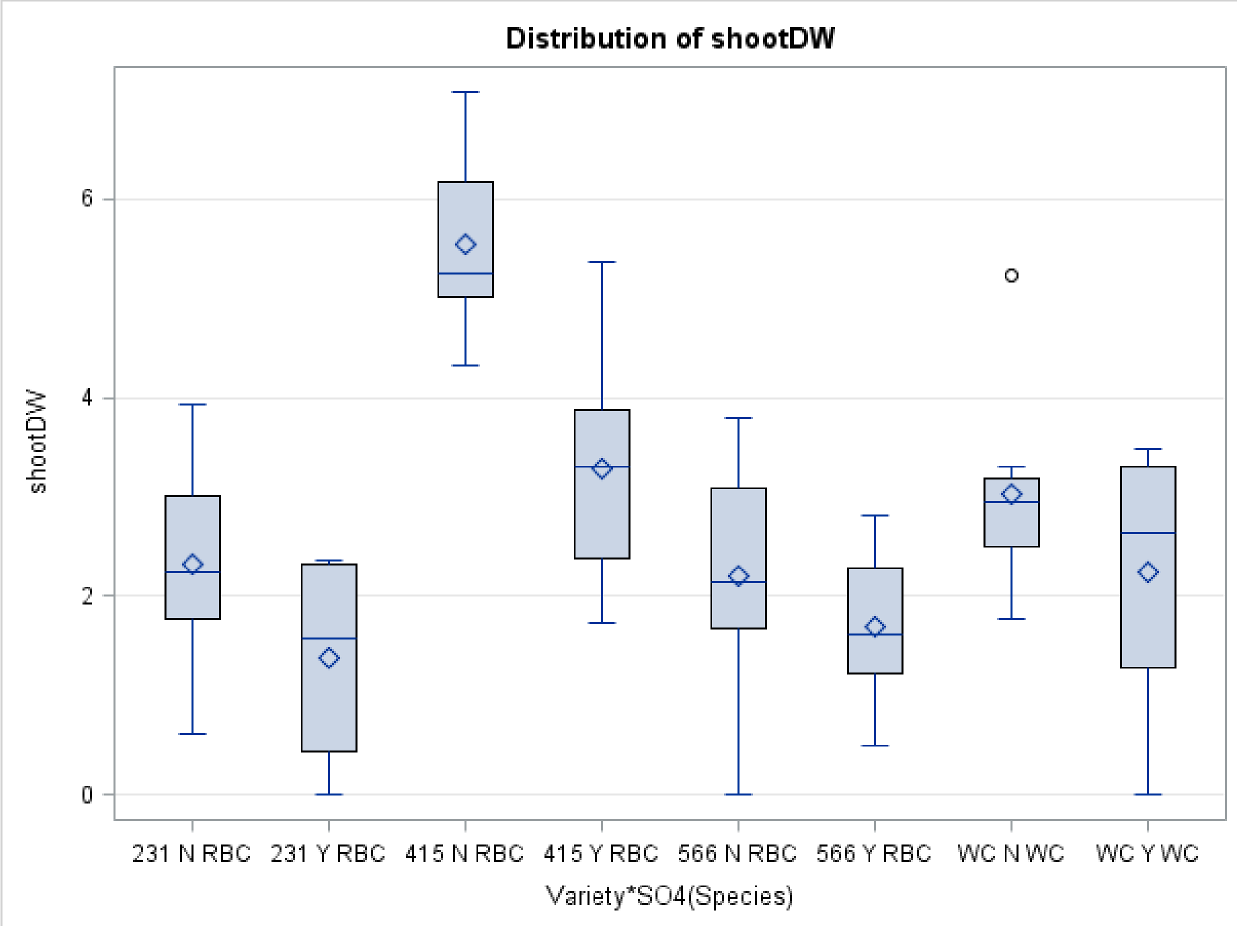
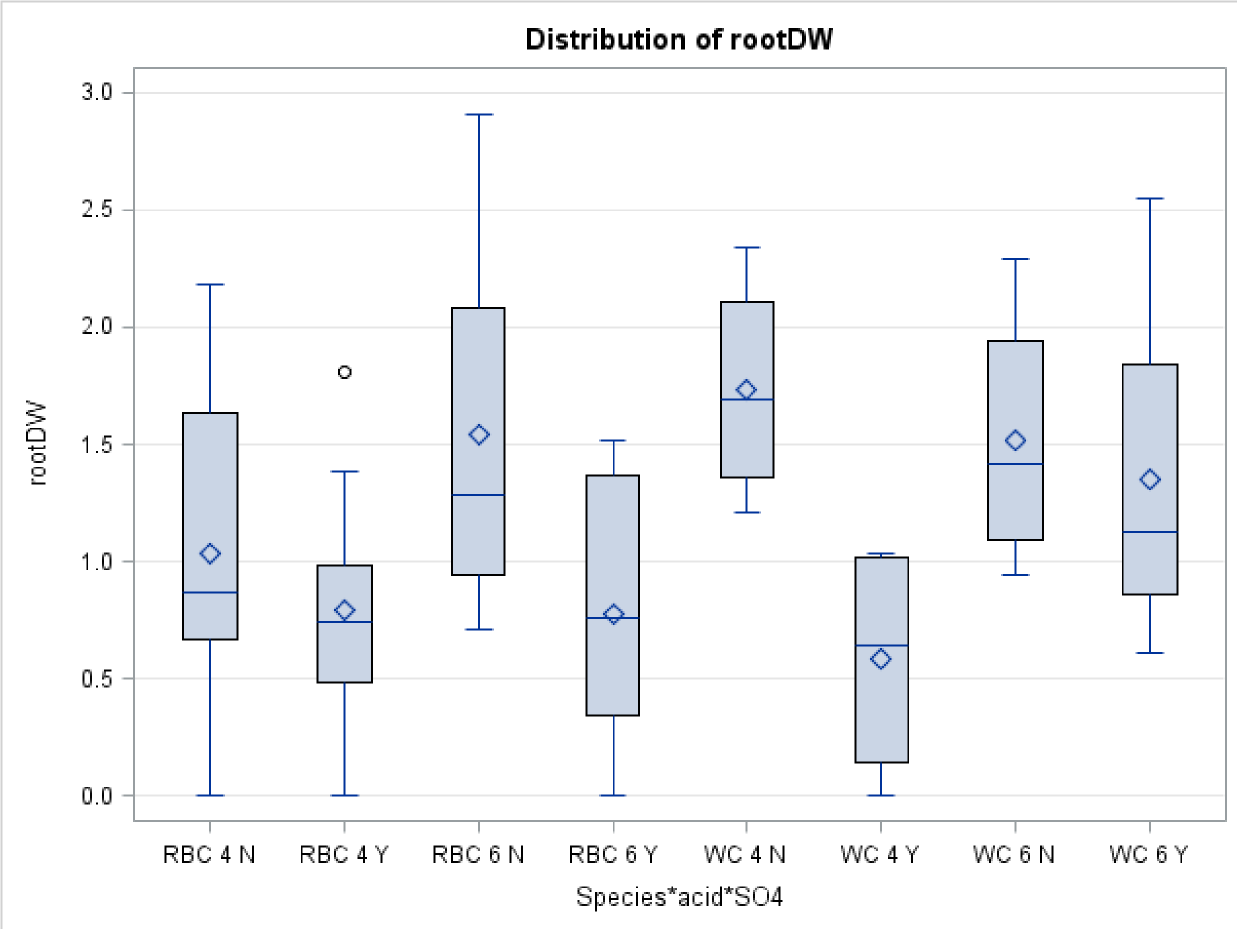


Figure 2.6. Experiment 1. Root dry weight species by acid by sulfate interaction (p=0.0031)



Chapter 3: Morphological characterization of Bluegrass and Appalachian running buffalo clover accessions

Introduction

Morphological variation among and within populations of running buffalo clover (RBC) is a key determinant of the ecology of this species. While genomic information might assist in species relatedness delimitation, no morphological characterization of populations and regions exists for RBC (Crawford et al.. 1998). Determining inter-population and intra-population genetic diversity and divergence through morphology might practically inform conservation and research strategies. With constant population discoveries outpacing the speed of genomic data collection, morphological analysis and characterization serves as an immediate solution to conservation managers.

The Bluegrass and Appalachian regions presently described by the USFWS are based on the existing ecological differences, such as location, soil conditions, and botanical community, among sites and some differences in population genetics between regions (Selbo et al 2015; Crawford et al.. 1998). Some differences in pH responses were observed between these two sections of the range, e.g. Bluegrass accession 415 had more growing points. However, results from the experiments performed in Chapter 2 suggest that KY-origin accessions performed similarly to one another (Hattenbach 1996; Chapter 2).

As a generalization, RBC seems more genetically similar within populations and more different among populations (Crawford et al.. 1998). The USDA accessions publicly available trace from the University of Kentucky's Norman Taylor and may trace back to breeding material; they might exhibit less variation (Taylor et al.. 1994; Quesenberry et al.. 1997). Three Ohio populations were sampled to produce Ohio RBC accessions. The available Ohio material comes from up to 25 genotypes per accession and is likely more variable. White clover is very phenotypically plastic, but RBC is likely less so; however, the range of habitats, including RBC in the mowed lawns compared to woodlands suggest the existence of some phenotypic plasticity (Chapman 1983; Taylor et al.. 1994).

Studying morphological characters can identify unique populations for conservation purposes (eg improve gene banking procedures, monitor ex situ genetic conservation inexpensively, and identify optimal crosses for artificial population crosses). Producing novel gene pools for transplantation or genetic enhancement might serve to reconnect persisting remnants with genetic migration (Smith 1998; Taylor et al.. 1994). Such genetic bridges improve long-term persistence. Additionally, identifying genetic drift in curated populations relative to wild populations in terms of morphology and phenology might yield useful insights into drift effects on semi-domesticated lab populations, as well as preventing such effects.

Objectives

I sought to determine the different morphological responses from five USDA accessions of Kentucky plant material and three Ohio populations: Boch Hollow, Shawnee Lookout, and Miami Whitewater Forest. From these observations I hoped to document phenotypic plasticity and note any differences between the Bluegrass accessions [USDA RBC accessions, Shawnee Lookout, and Miami Whitewater Forest] and Appalachian [Boch Hollow] accession. The objective of the present study is to characterize the morphological variance of running buffalo clover across its Ohio and Kentucky range in an attempt to determine conservation priorities as well as evaluate novel germplasm for ex situ conservation. Previous work evaluated the genetic diversity of the species, but not the potential phenotypic plasticity; agronomic measures could pick up such morphological variability (Crawford et al.. 1998). Through the measurement of accepted taxonomic characters, underlying genetic diversity might be studied. As such I observed canopy height at the plant's base, stolon length and counts per plant, as well as flower and bud counts, characters used in taxonomic comparisons of native clover species (Chapel and Vincent 2013; Chapman 1983, [and Anderson] 1987a, 1987b). Ascertaining ideal delineation characters within the species improves monitoring efforts (Perkins 2015). Understanding the anatomical and physiological differences between populations and genotypes, would poise future genetic studies to explore differences between morphological groups.

Methods

The study involved comparing USDA accessions—originally from Norman L. Taylor's Kentucky collections—and Ohio wild populations; the Bluegrass regional identity of these accessions was verified by contact with an original collector, Dr. Julian Campbell (Dr. J.J.N. Campbell, pers. comm). Due to the complex regulatory environment and logistical delay material from West Virginia, Missouri, or Kansas were not included in the study.

Plant material came from stolons propagated in early December 2017. These stolons were grown in Sungro professional mix within root trainers (Sun Gro Horticulture Agawam, MA, United States of America). The material was vernalized for a half month during late January in a polyhouse before planting in the Ohio State University Kottman Hall greenhouse, Columbus, OH.

Chronology. On February 6, 2018, the plant material from 5 USDA Kentucky accessions and 3 Ohio wild populations was planted into 34 totes of Sungro Professional Mix. These totes were grown in the Kottman Hall greenhouse with supplemental lighting from February 6 onward and with initial hand watering.

An initial stolon count and measurement, crown canopy height, and flower/bud count took place 6-8 March. Stolon counts per plants and measurement were selected as stolon growth is a taxonomic character of value that distinguishes running buffalo clover from other North American species (Chapel and Vincent 2013). Measurements were repeated on 14 March, and 3-4 April, 2018. Phenology of inflorescence production was recorded over time for the duration of the study with stolon counts and stolon lengths.

Plants were destructively harvested for shoot mass on 5-6 April, 2018. Plants were dried for 1 wk (55^{o}C) with measurements commencing 12 April, 2018.

Experimental Design. The experiment comprised 20-22 genotypes of eight plant treatments (five KY accessions, three OH populations) in a RCBD design with unequal replication (2-3 replications). The experimental unit was a pair of totes [24 plants (genotypes) in total] per accession. There were unequal numbers of replicates and genotypes; in total there were 840 plants (35 totes x 24 plants per tote). Measurements included stolon counts, canopy, bud counts, and active flower counts were taken 6-8, 14 March, and 3-4 April 2018. Shoot mass measurements took place at the end of the study, 5-6 April 2018. Due to the death of more than 10% of the 732 and 415 accessions, these accessions were omitted from statistical analysis.

Analyses were conducted in R (version 3.4.3) (R Core Team 2013), using the ‘lmer’ option for analysis of a mixed model (Appendix 2). Replications and genotype-within-accession were analyzed as random effects, and plant material (accession or population) was analyzed as a fixed effect. A Shapiro test confirmed non-normality for all response data. Kruskal-Wallis analyses of the data were conducted as an alternative to ANOVA as a nonparametric equivalent. LS means and Fisher's Least Significant Differences (LSD’s) were calculated. Boxplots were al.so generated as a non-parametric view of the data.

Results

ANOVA yielded significant results for Accession effects on stolon count, canopy, bud, and flower counts. (Tables 3.2-3.5) (Figures 3.2-3.5)

Genotypes exhibited similar patterns of growth between replicates. The canopy and stolon count responses exhibited similar accession and nested accession/genotype effects (Figures 3.2-3.3). This pattern was less strongly observed across all characters (Tables 3.2-3.5).

Plant material performed differently at the accession level, though variance within populations was generally similar. Many small plants flowered at least once, and so some flower performance and stolon growth co-occurred (Figures 3.4-3.5). The range of response values was similar across accessions (Figures 3.2-3.5). Accessions obeyed a moderately consistent ranking across all responses, including growth (Tables 3.2-3.5). Population differences existed among but not between the five Bluegrass accessions and the one Appalachian accession, Boch Hollow. Boch Hollow behaved comparably with 231 and with Miami Whitewater Forest material (Figures 3.2-3.5). Shawnee Lookout and 565 outperformed the other accessions in all responses measured (Figures 3.2-3.5). Miami Whitewater Forest was the weakest performing accession overall (Figures 3.2-3.5; Tables 3.2-3.5).

Discussion

Kentucky vs Ohio plant material

566 and SL were generally similar. Although from different states, the close proximity of these populations (southern Ohio and northern Kentucky) makes it possible these populations could be genetically similar. The accession/populations exhibited high variability, and in general, variation was greater within a population than was variation among populations (Figs 3.2-3.5)

Bluegrass vs Appalachian plant material

Boch Hollow was the only Appalachian accession available for the study. Presently there appears to be no significant differences in all responses measured between the Bluegrass and Appalachian regions. Boch Hollow plants grew similarly to the least productive accessions from the Bluegrass Region. Additional accession comparisons might yield a different result, but presently it appears that the plants from these two different ecological regions are not much different from one another phenotypically.

This study is the first to compare phenotypic differences between Kentucky and Ohio plant material. Flowering was earlier than natural field populations, since plants had been stratified in the tunnel house, and when brought inside the warm greenhouse, the plants were induced to flower with supplemental lighting.

With access to study populations, researchers might produce material worthy of field introduction. Even without population genetic information, vegetative propagation can substitute for field fecundity in improving population sizes (Sparks and Barker 2013). And with stronger tools for field characterization, agencies might assign priorities to specific field populations.

The similarity of variability in accession responses supports existing genetic and anatomical understanding of the species. 2-3 populations had generally poor vigor. This might have been related to disease (i.e. virus). Clearly, the wild populations and cultivated accessions varied in the extent to which they were affected by the virus. This variability al.so supports the possibility of genetically limited populations to explore several growth strategies. Plants can explore a growth and/or reproductive strategies within a range of phenotypic expression. RBC exhibits a narrow, but functional range of phenotypic plasticity. Understanding phenotypic responses in management decisions might improve population persistence by supporting stolon production and/or flower formation outside the traditional management around the May-June flowering window.

Given the limited genetic surveying available within RBC research, connecting phenotypic data to genetic studies should remain a pertinent goal to understanding behavior of the species.

Table 3.1. Populations and accessions sampled

Population/accession	Year collected/produced
566, Kentucky	2011
565, Kentucky	2011
732, Kentucky	2011
415, Kentucky	2011
231, Kentucky	2011
Shawnee Lookout, SL, Ohio	2017
Miami Whitewater Forest, MWF, Ohio	2017
Boch Hollow State Nature Preserve, BH, Ohio	2017

Table 3.2 Canopy height (cm) LSDs March 15, 2017

565	4.81 a
SL	3.54 b
566	2.75 bc
231	2.50 c
BH	2.37 c
MW	1.41 d

Table 3.3 Stolons/plant LSDs March 15, 2017

SL	4.82 a
565	4.57 ab
231	3.11 bc
566	2.86 bc
BH	1.98 cd
MW	0.93 d

Table 3.4 Flowers/plant LSDs March 15, 2017

SL	0.92 a
565	0.74 ab
BH	0.36 bc
566	0.33 bc
231	0.27 c
MW	0.10 c

Table 3.5 Buds/plant March 15, 2017

565	0.42 a
SL	0.41 a
BH	0.28 ab
566	0.11 ab
231	0.07 b
MW	0.06 b

Figure 3.1 Initial inflorescences in the study. Shawnee Lookout plant material March 5, 2017

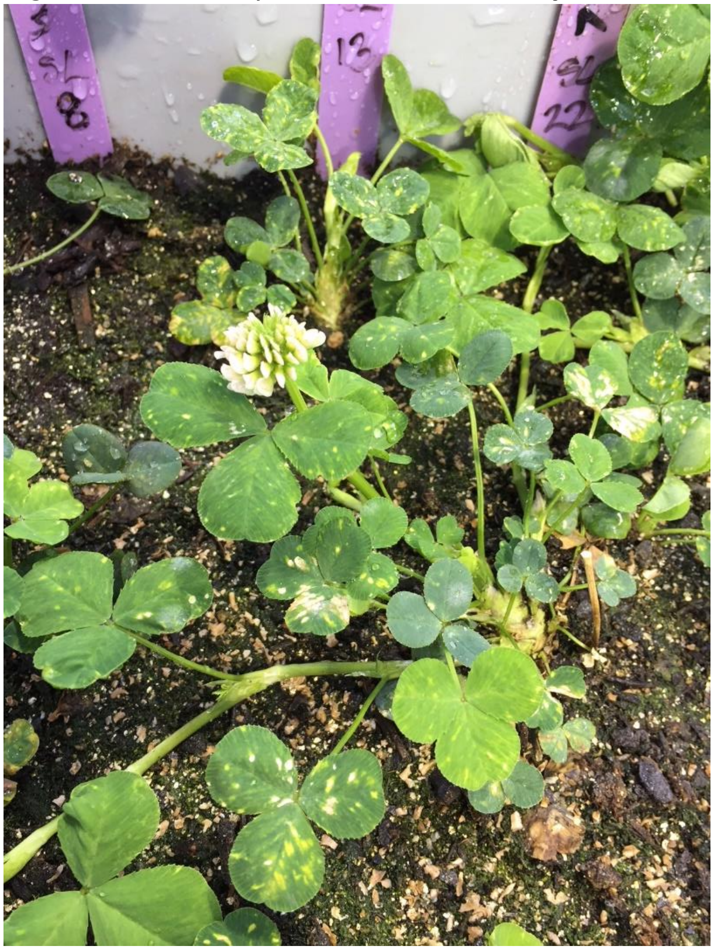


Figure 3.2 Stolons/plant March 15th observations

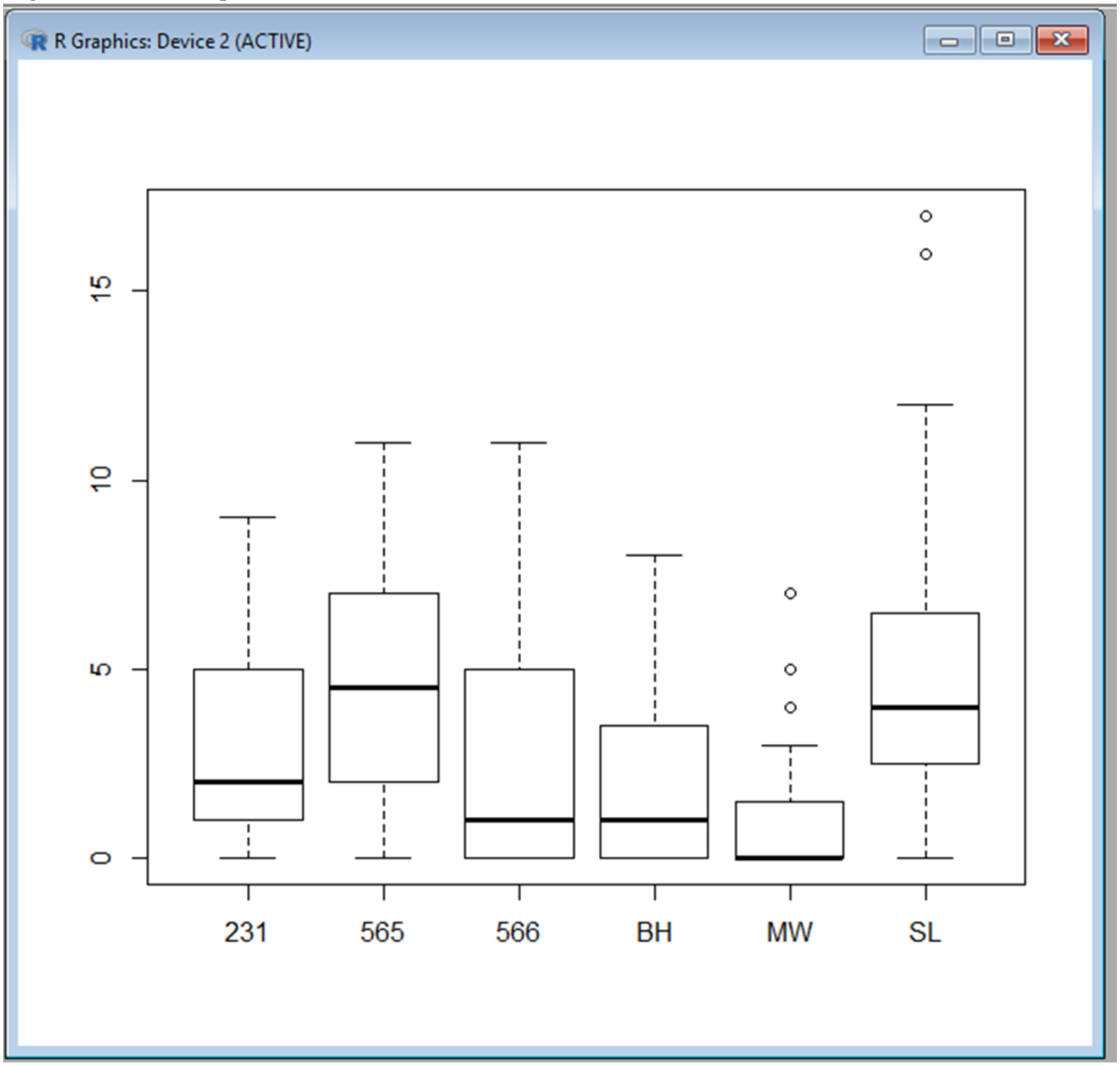


Figure 3.3 Plant height (cm) March 15th observations

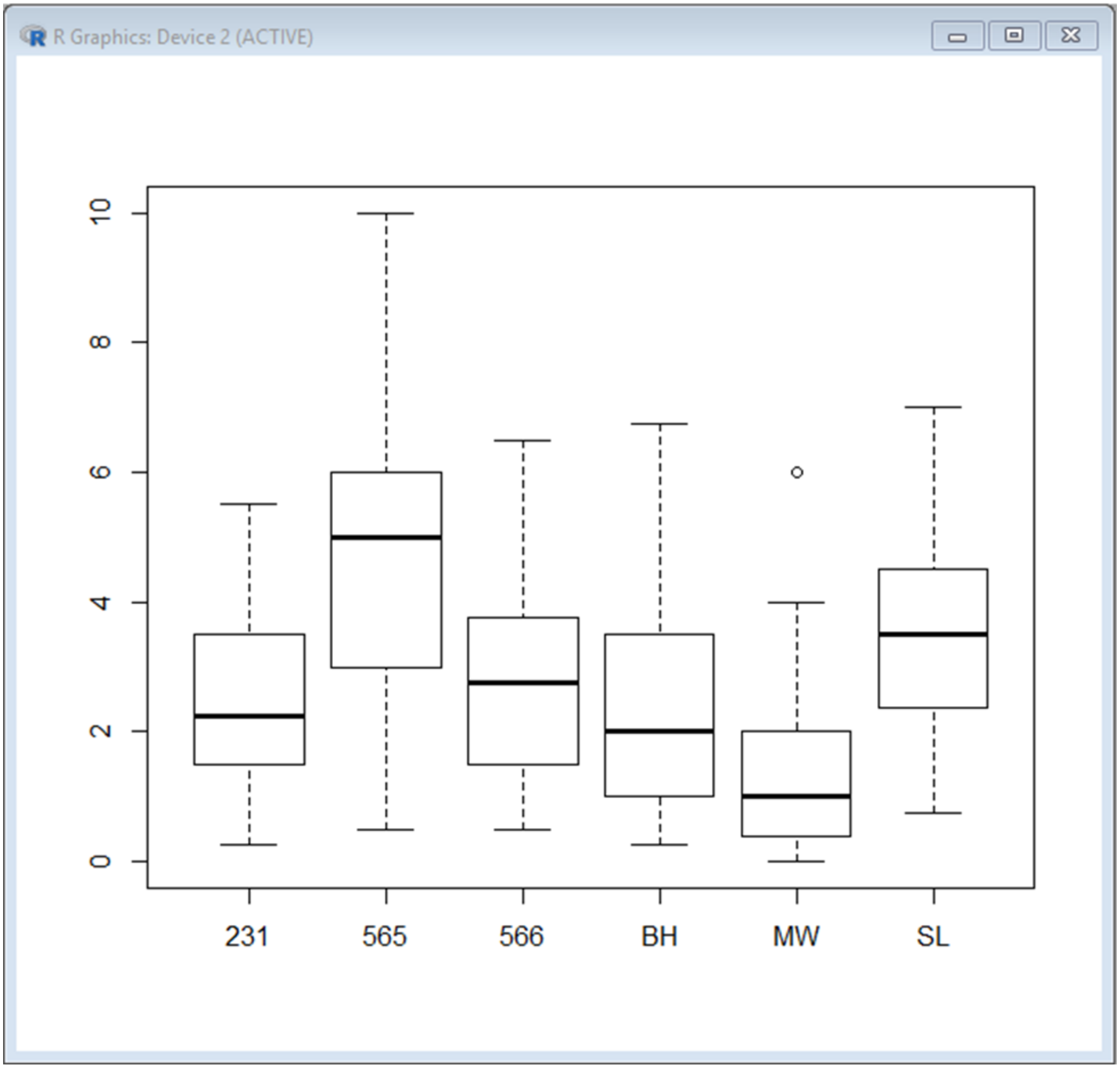


Figure 3.4 Flowers/plant March 15th observations

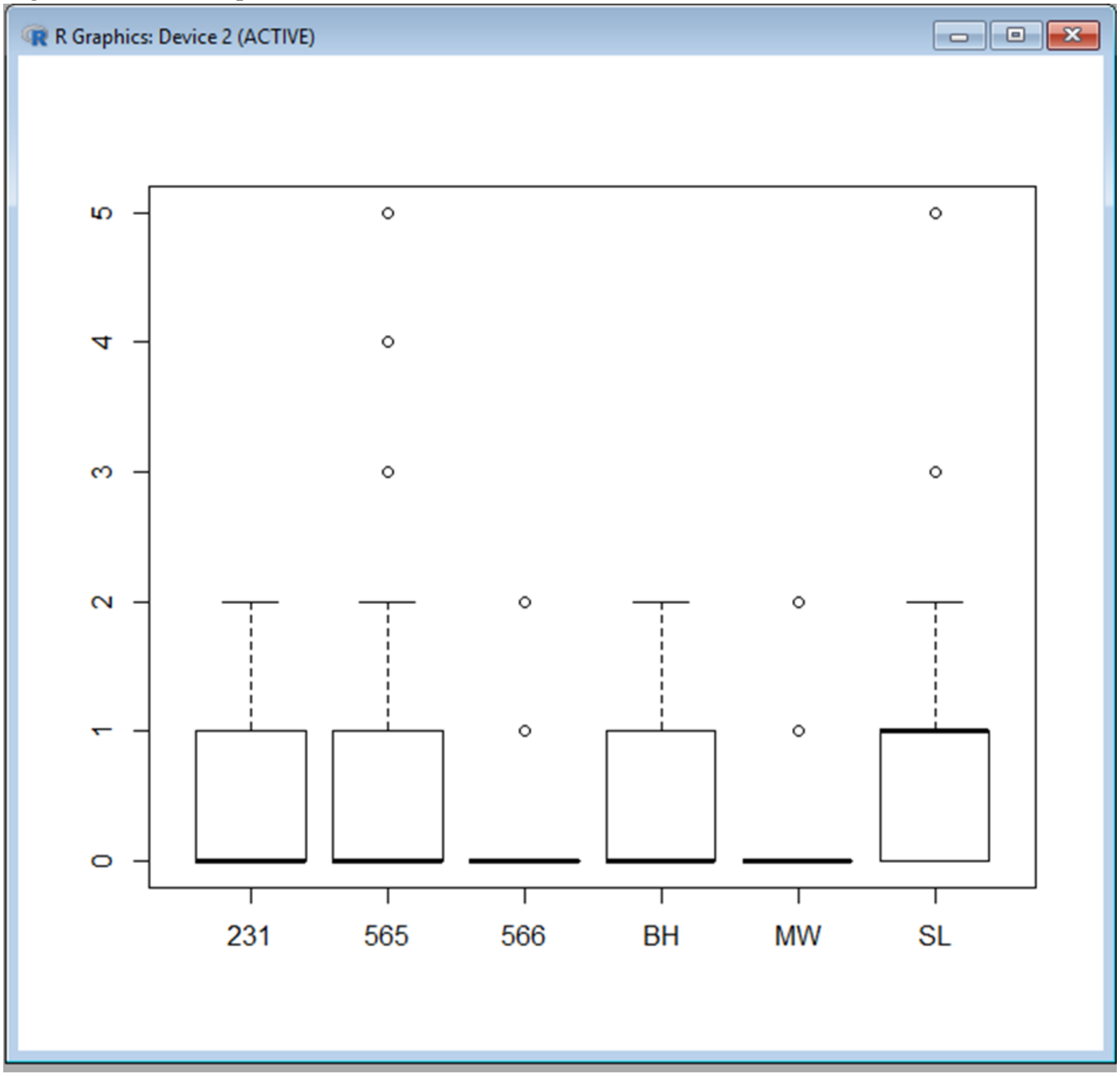
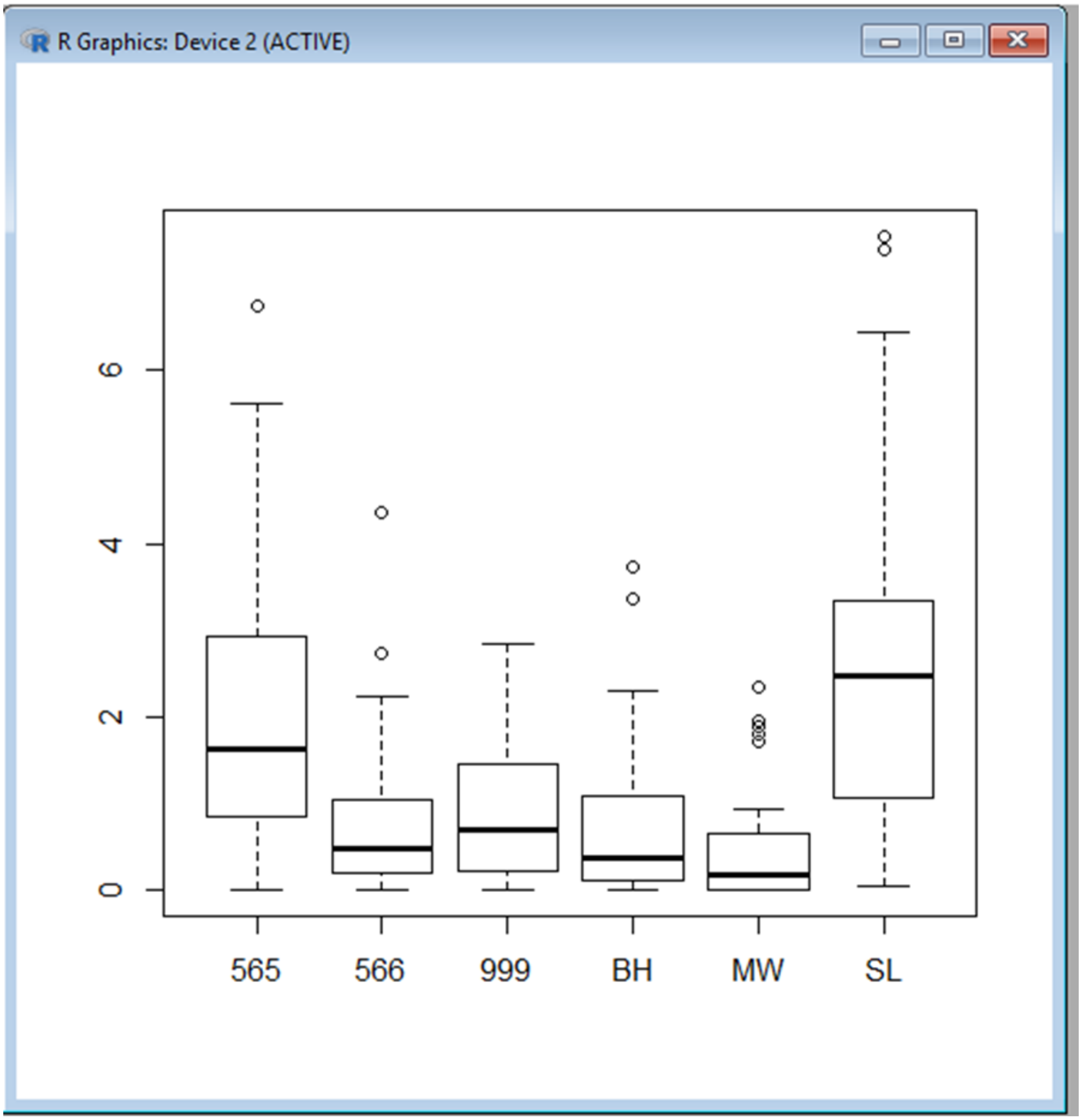


Figure 3.5 Shoot (g/plant) April 6th results



Chapter 4: Optimization of transplantation protocols for running buffalo clover

Introduction

Over the past 30 years, researchers and conservationists discovered and protected wild populations of running buffalo clover (RBC) (*Trifolium stoloniferum)*. Throughout these many years of various observations and field studies, few researchers studied the species in cultivated settings (Smith 1998; Barker and Sparks 2013; Perkins 2015). Despite limited seed banking by the Missouri Botanic Garden and the U.S.D.A., few biologists or agronomists call for ex situ conservation or repatriation studies (Yatskievych 2006).

In emergency cases where field translocation is required due to habitat threat, authorities report high fatality rates, as high as 90% (Gardner et al. 2017, pers. Comm., March 28, 2017; October 2017). Such low survival rates in these cases threaten the already small populations of RBC, and might further reduce the genepool than at current low levels. Occasionally, field populations of RBC require to be transplanted due to threats at one location, such as an alternative land use. Current transplanting protocols include specification of: i) optimum timing (to avoid plant establishment during the hot/dry conditions of summer), ii) ensuring a large root ball, to minimize root disturbance, and iii) the new area to be as similar as possible to the original location. (Gardner et al.., pers. Comm., March 28, 2017).

Greenhouse intervention, as an interim step during repatriation, would address developing ideal protocols for the following:

i. Collection protocols for nondestructively sampling wild populations (Sparks and Barker 2013)

ii. Ascertain the ease of propagating wild material relative to research germplasm

iii. Transplantation techniques that improve the survival of propagated specimens in the field as well as work within pre-existing regulatory and monitoring schemes (Perkins 2015; Smith 1998; McKenzie et al. 2018)

iv. Might allow for clonal increase by vegetative propagation in the number of plants (greater than one as in direct plant relocation), and reduce the likelihood of an individual genotype from becoming lost.

v. Might allow for ‘surplus’ clones being available for ancillary research without threatening to the existent population (see Chapter 3)

As one of the few research groups investigating the species in the greenhouse, the present study seeks to determine optimal transplantation protocols as well as the role of greenhouse intervention in *in situ* and *ex situ* conservation of running buffalo clover. The study investigated whether greenhouse intervention for RBC transplants could improve field survival; plant size/age, root media, and field site will affect transplant success of greenhouse raised plants; and field transplanting be improved with use of a one-time starter fertilizer.

In developing optimal transplantation, researchers, and conservationists might take advantage of edaphic and ecological data in selecting sites for new populations in repatriation schemes (Perkins 2015; Burkhart et al. 2013; Hattenbach 1996). Improving repatriation with this crucial step in propagation should increase success rates and allow researchers to factor in additional population biological information into such projects; logistical challenges overcome, larger genetic and ecological questions in restoration might be addressed (Becus and Klein 2002; Dart-Padover et al. 2016).

Objectives

I attempted to optimize transplantation protocols using greenhouse intervention for low-input conservation efforts. In consequence, I aimed to propagate field-sampled stolons in sufficient quantities to support field transplanting.

Methods

Stolon collection and greenhouse intervention:

Following official permitting with the Ohio Department of Natural Resources (ODNR), the U.S. Fish and Wildlife Service (USFWS), and the appropriate landowners, 25 stolons were collected from three populations of interest. These stolons were collected at the first rooted node and placed in general greenhouse media. Population sources achieved A or B ratings from the USFWS and ODNR, determined from census population size (200-1000 plants =B, 1000+ plants =A) (Table 4.1). These population ratings limited the potential impacts to smaller populations and served as ideal sites for future repatriation studies due to their larger areas and the support of their management agencies. After propagation in the Kottman Hall greenhouse in Sungro professional mix (Sun Gro Horticulture Agawam, MA), stolons yielded plant material which went into 0.1 m^2 pots or root trainers for prescribed propagation periods of 3-4 months or 3-4 weeks respectively. Plant material generated over 3-4 months of growth in 0.1 m^2 pots and plants generated over 3-4 week in root trainers were both grown for planting at sites nearby the pre-existing source populations. Plants were acclimated to fall weather conditions in a limit-heated tunnel house (minimum temperature -10^{o}C, Howlett Polyhouse C) with natural light (8-10 hr daylength) during 15 December 2017-April 2018. Plants were watered weekly, and fertilized each month.

Transplantation:

Plants were replanted into their three source locations: Boch Hollow, OH, in mid-October 2017, and Shawnee Lookout and Miami Whitewater Forest in early-November 2017. At each site a 1m x 1m grid of equally-sized planting holes was dug after inspecting each site to ensure it was free from pre-existing RBC plants (Table 4.2). Plants went into the assigned positions as per the pre-assigned randomization, planted into their hole with or without 19-19-19 fertilizer and watered with 250-500 mL to limit transplant shock. A plot map was generated using Microsoft Excel 2013, with plants being randomly assigned to positions and treatments within a planting order. This planting order allowed for randomizations that wouldn't be beholden to a specific plot grid.

Plants remained unmanaged until the spring, at which point standard management regimes—seasonal mowing, plant censuses, canopy thinning—for the existing populations were carried out on the sites (Table 4.2).

The Shawnee Lookout and Miami Whitewater Forest sites were situated adjacent to the remnant populations. Both sites shared a history of RBC presence, but lacked plants at the time of site selection. Both sites had similar soil properties to their source populations. The Miami Indians and their predecessors constructed the earthworks present at Shawnee Lookout; the site exists on the Miami Fort which once served as a 20th century campground (Marjorie Becus, pers. comm.). The Miami Whitewater Forest site lies along a recently installed asphalt bike path through a lowland woodland. The source population grows within the mowed shoulder. The Boch Hollow site was planted in a hollow 1 km from the source population. This hollow had no history of RBC populations; however, at some point between logging and the reforestation of the site, pasture covered most of the property. Presently the old pasture is maintained on the hilltops whereas the forest has recolonized the lower elevations within the hollows. The Boch Hollow site is situated on a logging roadbed abandoned sometime in the early 20^{th} century (Levi Miller pers. comm.). All three planting sites were adjacent to mature black walnut trees.

After initial measurements, the sites persisted through the winter until a spring measurement in April 2018. The measurements in spring worked within the limitations of a thesis deadline, and thus the flush of growth the species characteristically exhibits in May/June didn't factor into present publication.

Experimental Design: The experimental design at each site consisted of a 20 (or 22) x 2 x 2 factorial of 20 or 22 genotypes, two levels of plant size (large, 3-4 months old, and small 3-4 weeks old), and two levels of fertilization (no fertilizer, vs 2.5 g/plant split at the base of the plant, and spread on the surface). There was a variable number of plants for each genotype, which allowed for a variable number of 2-4 replications per treatment, in a completely randomized arrangement. The experimental unit was a single plant. Genotype was treated as a random effect, whereas plant size and fertilization were treated as fixed effects. This mixed model was applied to each population separately.

Measurements of stolons per plant and stolon length were made at planting (in fall 2017) and in the spring 2018 (Table 4.2). Statistical analysis was conducted separately for each site, using R (Appendix 3).

Results

The weather during fall was good for establishment, being warmer than average with average rainfall. Winter had some cold weather, but was considered average. The temperature during March and April was colder and wetter than average.

Boch Hollow. Fertilizer and size treatments were significant in both the stolon counts and lengths produced at the Boch Hollow site. Moderate grazing was noted on several of the larger plants, however only a few plants were dug up from their original planting holes. Surrounding vegetation hadn't overtaken the site, and leaf litter was moderate under the dappled-shade canopy (Figure 4.1). The Boch Hollow population had the highest survival rate of all three sites (Table 4.2). Stolon counts per plant and total stolon length responses were both significant for plant size in both the fall and spring (Table 4.3, Figures 4.4-4.15).

Shawnee Lookout. Moderate grazing was observed at the Shawnee Lookout site. Two plants were noted as dug up at the site, and no plants were missing; the remains of deceased plants remained in the planting holes (Figure 4.2). Some moisture pooling was noted in the planting holes. Stolon counts per plant and total stolon length responses to plant were both significant in fall; in spring fertilizer and size effects were al.so significant (Table 4.3, Figures 4.4-4.15).

Miami Whitewater Forest

Over the unusually cold and wet winter, the stream adjacent to the site flooded several times to around 2 m depth. In that time, around 50% of plants washed out of planting holes and were considered lost (Figure 4.3). Remains of 20% of dislodged plants were identifiable, and could be replanted. The remaining 30% of plants not be found, even in searching outside the planting site in the surrounding woods and creek. This site effect was not compared to other sites directly, but did explain the markedly lower survival rate of Miami Whitewater Forest compared to the other two sites. Stolon counts per plant and total stolon length responses were both significant in both the fall and spring (Table 4.3)

Discussion

While fertilizer-by-size interactions were almost significant in all populations, only Shawnee Lookout had a significant interaction (Table 4.3). Larger plants responded positively to fertilizer whereas smaller plants responded negatively to fertilizer at Shawnee Lookout (Table 4.3; Figures 4.4-4.15). The benefits of the fertilizer treatment may linger into the future and barely appear in the present analysis. Smaller plants seemed to benefit relative to larger plants, which were grazed more heavily. Due to the browsing behavior of deer, as well as their high concentration around the Hamilton County area, some of the plants dug out from the holes might have simply been deer tugging plants by the stolons (Levi Miller pers. comm.; and Zuri Carter pers. comm.).

Experimental population failures from the repatriation program in Missouri lead many to discount the possibility of larger repatriation programs in other states (Smith 1998). Using material from across the eastern range, the Missouri Department of Conservation proved the logistical feasibility of transplantation, but such work failed to address the need to clone and save wild vegetative material in emergency transplantations. The eventual termination of the repatriation with the discovery of indigenous Ozark material limited the study of long-term success in artificial populations (Smith 1998). Experimental populations in Kentucky persist with aggressive monitoring and reintroduction campaigns; ideally repatriation schemes might monitor populations until the usual population cycles observed in large, West Virginian populations approach a sustainable level (Perkins 2015; Burkhart et al. 2013). In a species of notable stochasticity, monitoring might eventually occur during the periodic disturbances artificially made across much of the range.

Long-term monitoring of introduced, experimental populations must address differentiation between introduced and indigenous populations. Despite short-term success, such transplantations must not only augment surviving wild material, but persist enough to maintain the gene pool. Momentary population increases without long-term improvements in population fecundity impede the aim of present recovery efforts: minimal intervention to support enduring populations (Becus and Klein 2002). Following present USFWS protocols in monitoring, but delineating between materials will support the validity of greenhouse intervention in the coming decades. Present success might be further supported with monitoring of population fecundity before and after similar interventions and repatriation strategies. Long-term experimental populations would allow researchers to determine ideal introduction numbers and study persistence across longer durations without adversely affecting the ecological or evolutionary fates of wild populations.

Differential propagation success between populations suggests potential challenges to extended ex-situ conservation programs. Namely, accessions will require individual treatment to maintain genetic diversity as well as avoid indirect selection for greenhouse conditions. Each population requires individual attention in managing intermediate or long term greenhouse population, and researchers must consciously avoid domesticating the clover through such conservation (Schoen and Brown 2001; see Chapter 5). Given the life history and selfing tendencies of the clover, such concerns must be considered, both in seed production as well as vegetative propagation (Franklin 1998; Dart-Padover et al.. 2016).

A continuing human and ruminant presence maintained the species well into the nineteenth century; running buffalo clover grows along the disturbed margins of the modern Midwest, where tractors, loggers, cattle, campers, cyclists, and artillery shells maintain half-acre patches. The importance of site selection, as evidenced by the physical disappearance of plant material at

Miami Whitewater Forest, cannot be argued strongly enough. Site selection that limits plant losses to catastrophic processes is encouraged in future plantings.

Specifically in Ohio, the present private and public research interest in RBC remains hindered by access to specimens, and the ability to generate experimental populations. Ohio's lack of large experimental management strategies and the differences in gene pools might make such observations inaccurate for Bluegrass material; however, future studies might address ideal management for Ohio's Bluegrass and Appalachian material using present empirical studies in Kentucky and West Virginia (Burkhart et al. 2013; Dart-Padover et al. 2016; Perkins 2015). In order to improve repatriation schemes, stakeholders need collaborate in planning new restorations and repatriations. The vegetative propagation success displayed here suggests that the state's total population could double with a few years; allowing such production within a revised USFWS framework would improve the present, perilous state of RBC in the wild and in the lab. At this stage in field observations, small fall transplants without a starter fertilizer appear to be the optimal specimens for transplantation. Transplantation work at Shawnee Lookout confirms a null hypothesis that bigger plants would be better at establishing than smaller, younger plants. The presence of stolons on transplants does not appear to be beneficial, since these can be readily lost by grazing deer and rabbits.

Table 4.1. Stolon collection from three Ohio populations.

Population	Location	Estimated census population score from U.S. Fish and Wildlife Service	Collection Date
Boch Hollow State Nature Preserve	Logan, Ohio Appalachia	A- (>500 plants)	23 May, 2017
Shawnee Lookout, Great Parks of Hamilton County	North Bend, Ohio Bluegrass	A- (>500 plants)	31 May, 2017
Miami Whitewater Forest, Great Parks of Hamilton County	Harrison, Ohio Bluegrass	B- (50-200 plants)	31 May, 2017

Table 4.2. Transplant Introduction and survival rates

Population	Date Introduced	Planted individuals	Date monitored	Survival rate
Boch Hollow State Nature Preserve	October 17, 2017	95	April 10, 2018	97.5%
Shawnee Lookout, Great Parks of Hamilton County	November 7, 2017	77	April 15, 2018	97.4%
Miami Whitewater Forest, Great Parks of Hamilton County	November 7, 2017	102	April 15, 2018	70.6%

Table 4.3. ANOVA results

Fall 2017	Treatment variable	F	Stolons/plant	F	Total stolon length
Boch Hollow	Fertilizer	0.12	NS	1.51	NS
	Size	107.05		79.72	
	Fertilizer*Size	0.02	NS	0.71	NS
Shawnee Lookout	Fertilizer	0.56	NS	0.0081	NS
	Size	87.01		68.21	
	Fertilizer*Size	1.51	NS	0.1067	NS
Miami Whitewater Forest	Fertilizer	1.23	NS	1.54	NS
	Size	69.42		37.64	
	Fertilizer*Size	1.81	NS	0.58	NS
Spring 2018	Treatment variable		Stolons/plant		Total stolon length
Boch Hollow	Fertilizer	0.0086	NS	2.04	NS
	Size	42.49		73.98	
	Fertilizer*Size	0.48	NS	3.93	NS
Shawnee Lookout	Fertilizer	0.424	NS	3.27	
	Size	66.56		43.98	
	Fertilizer*Size	0.0083	NS	2.83	
Miami Whitewater Forest	Fertilizer	6.67		2.69	
	Size	36.91		31.04	
	Fertilizer*Size	0.0016	NS	0.0007	NS

Figure 4.1. Planting at Boch Hollow (17 Oct 2017)

Figure 4.2. Planting at Shawnee Lookout (31 Oct 2017)

Figure 4.3. Planting the Miami Whitewater Forest site (Oct 2017)

Figure 4.4 Effects of size (0=small, 1=large) and fertilizer (-=0g/plant, +=2.4g/plant) on total stolon length at Boch Hollow, 17 October 2017

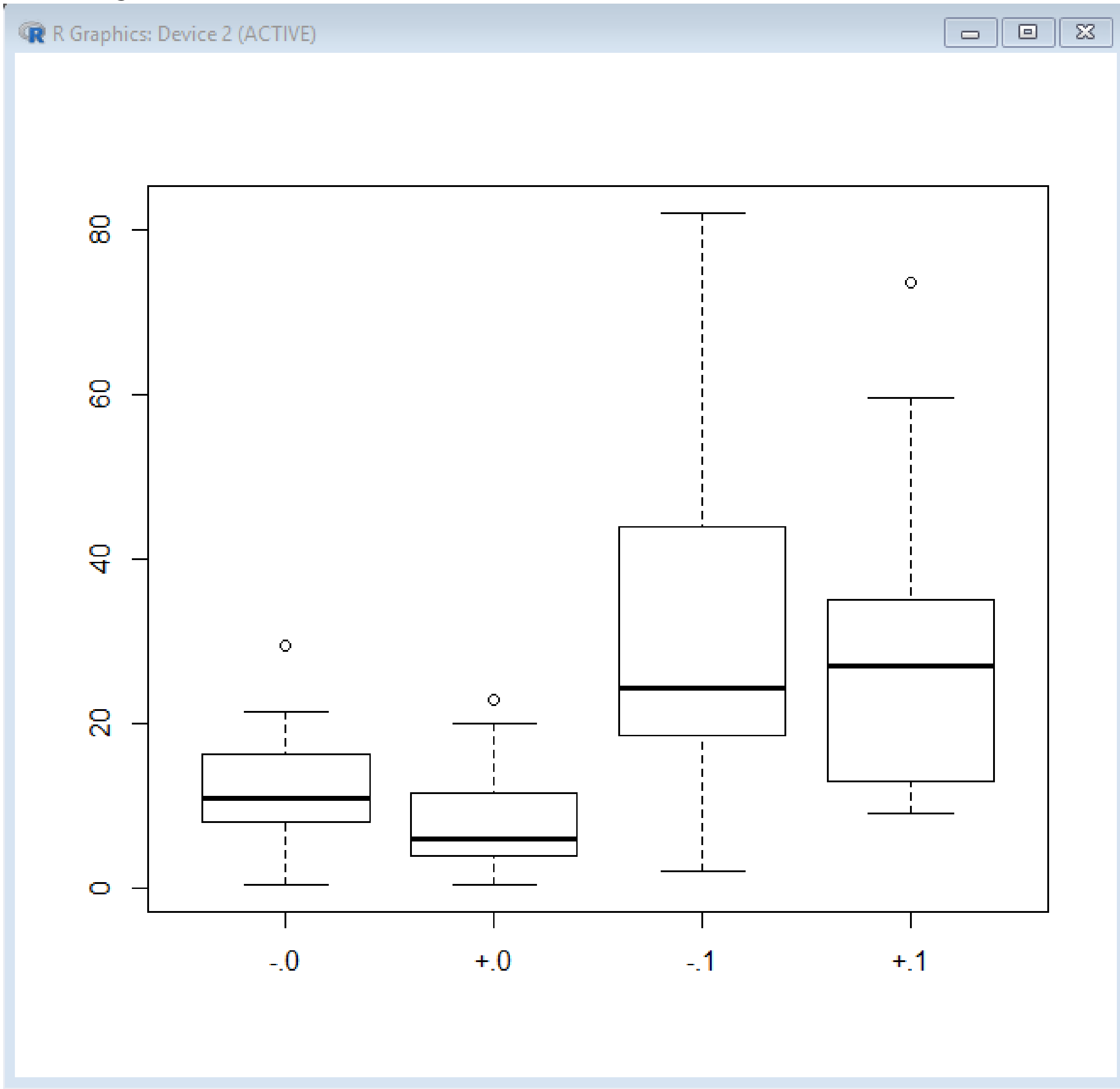


Figure 4.5 Effects of size (0=small, 1=large) and fertilizer (-=0g/plant, +=2.4g/plant) on total stolon length at Shawnee Lookout, 7 November 2017

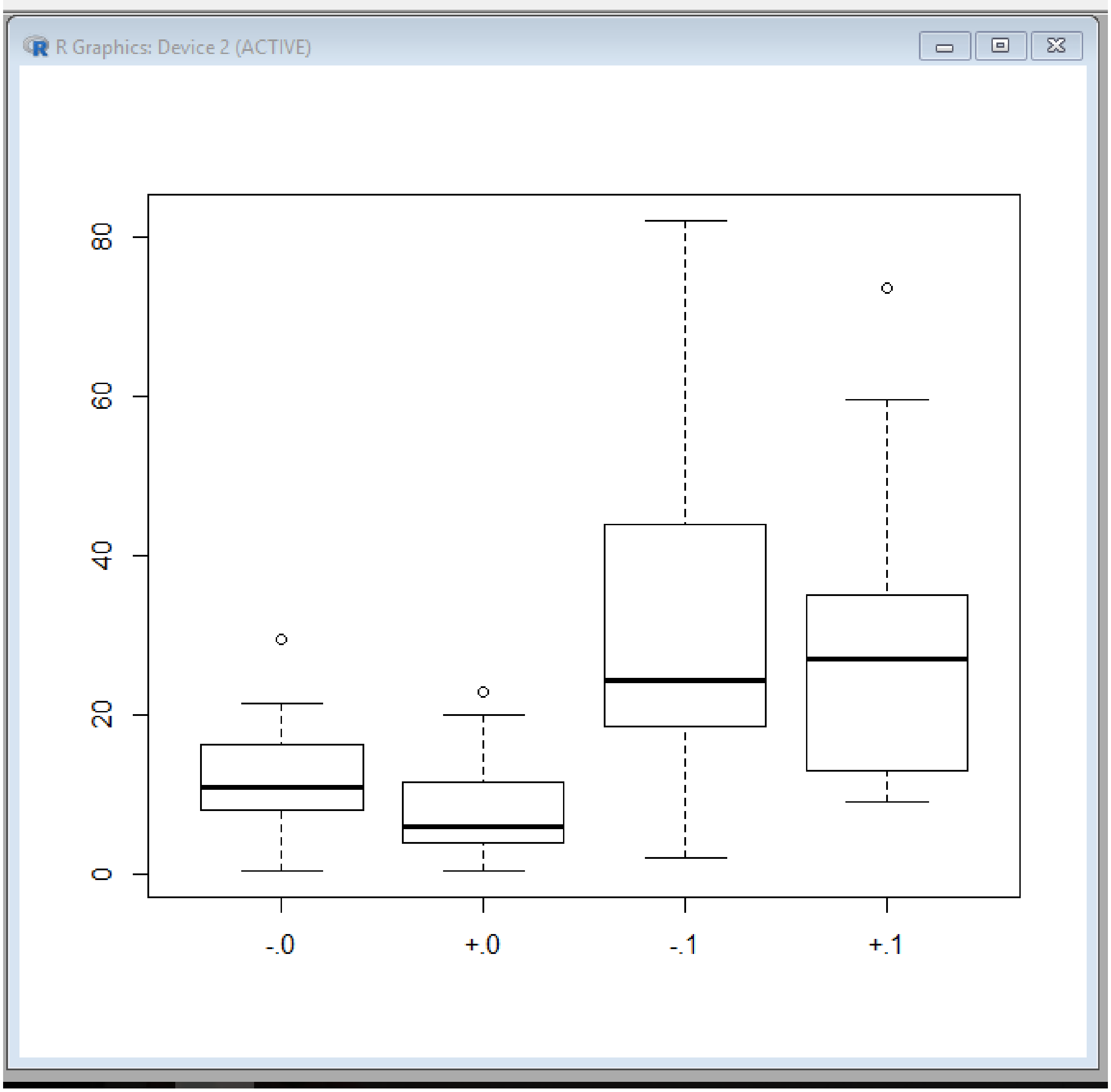


Figure 4.6 Effects of size (0=small, 1=large) and fertilizer (-=0g/plant, +=2.4g/plant) on total stolon length at Miami Whitewater Forest, 7 November 2017

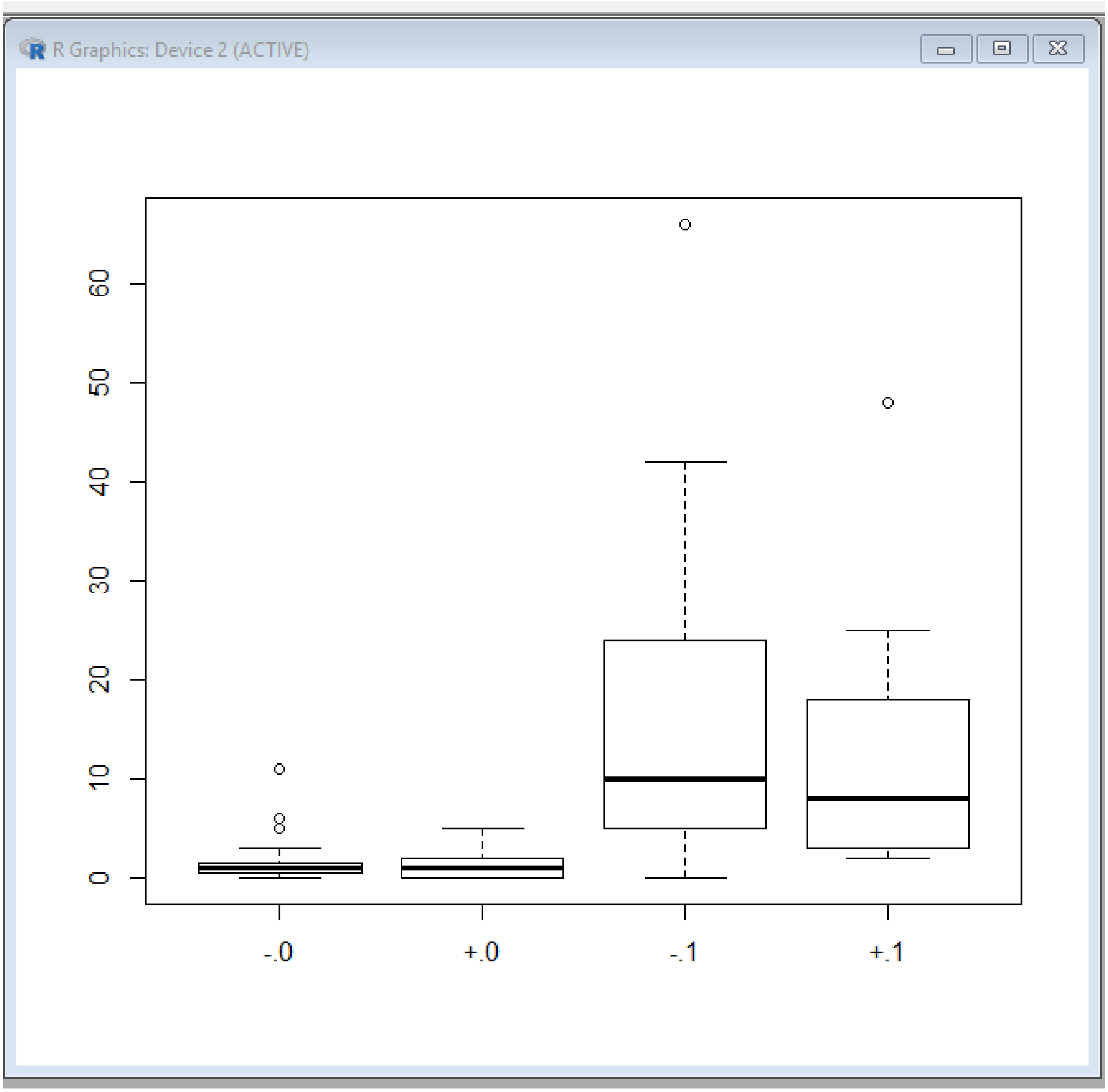


Figure 4.7 Effects of size (0=small, 1=large) and fertilizer (-=0g/plant, +=2.4g/plant) on total stolon length at Boch Hollow, 10 April 2018

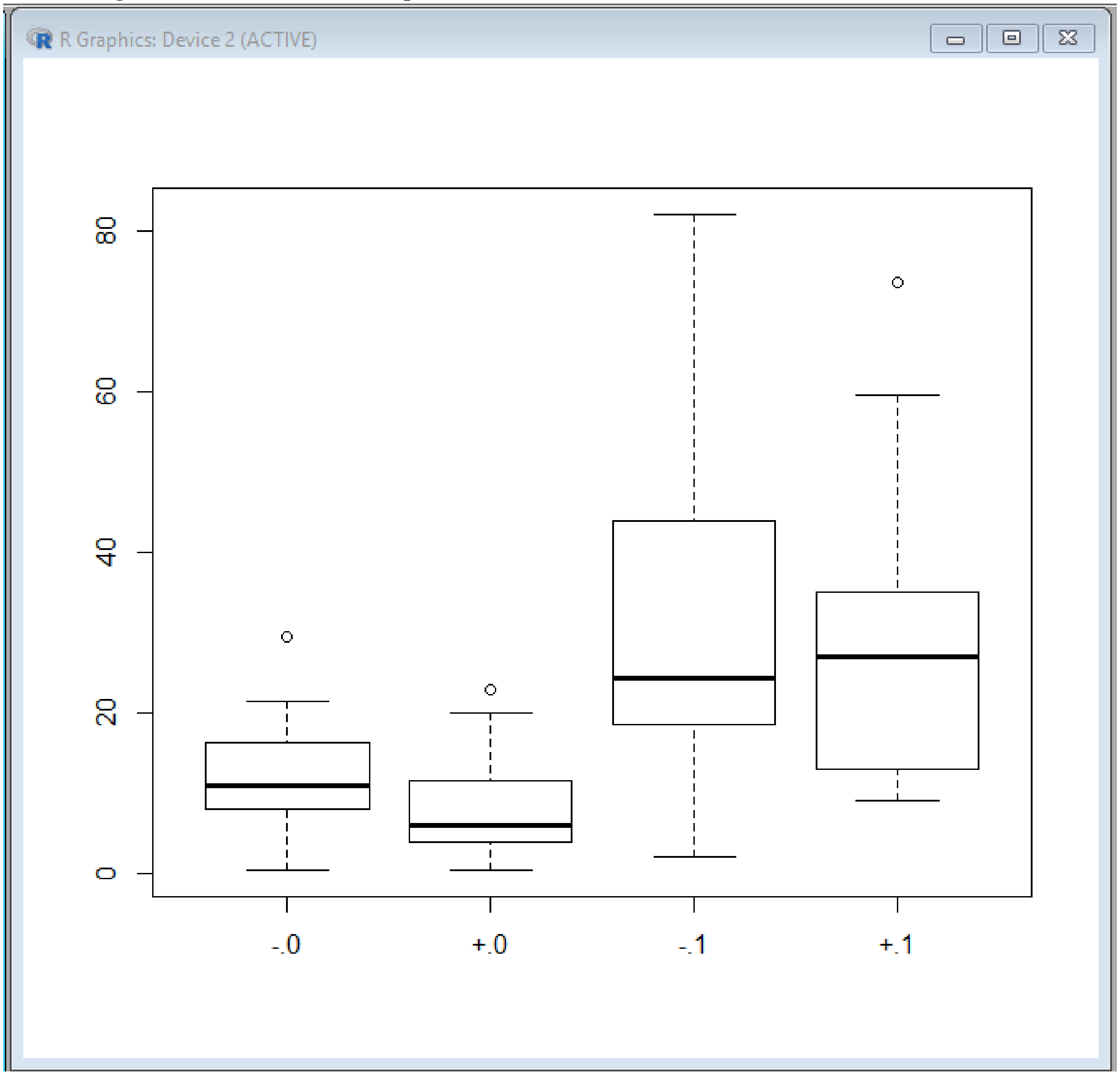


Figure 4.8 Effects of size (0=small, 1=large) and fertilizer (-=0g/plant, +=2.4g/plant) on total stolon length at Shawnee Lookout, 17 April 2018

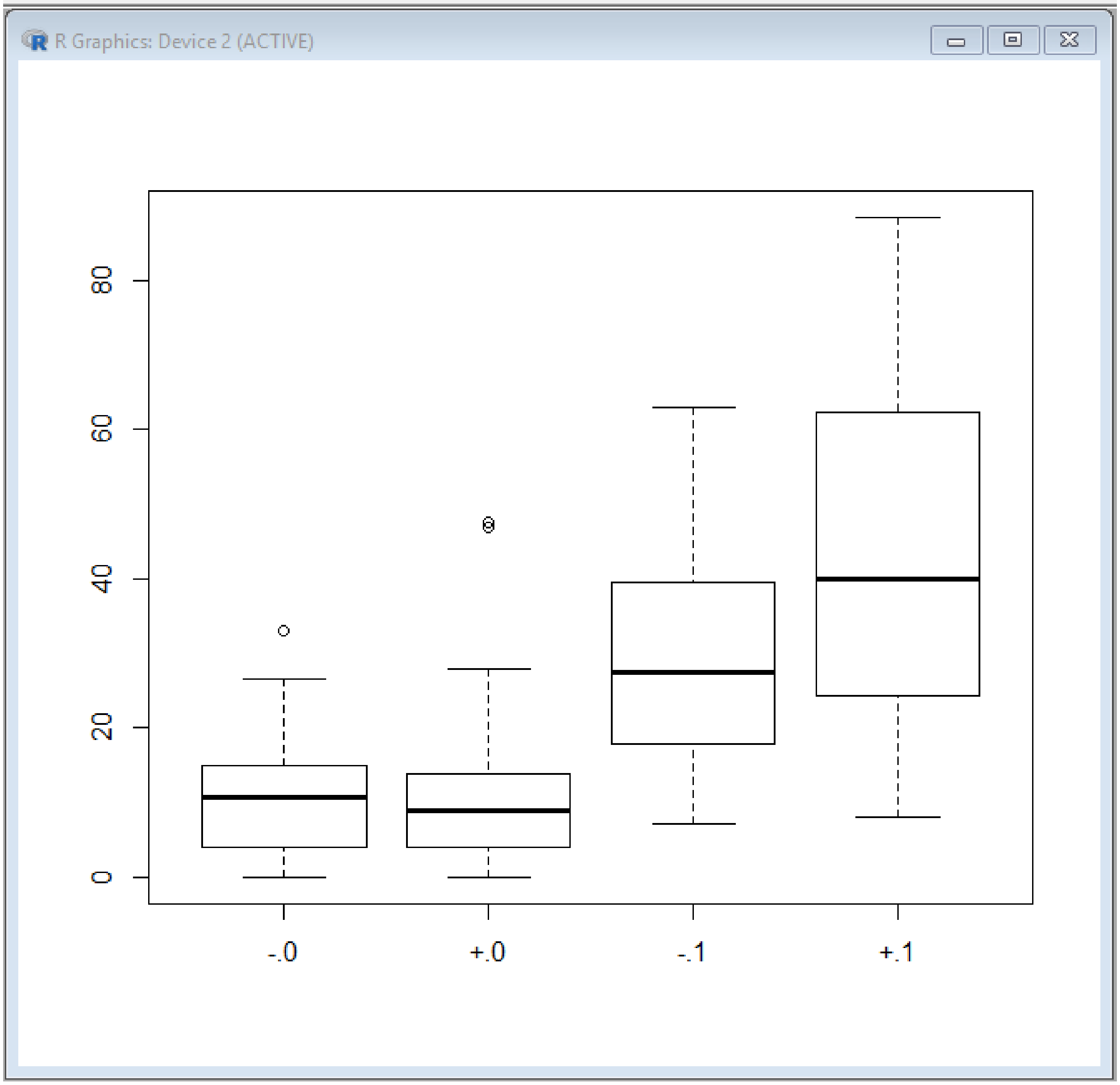


Figure 4.9 Effects of size (0=small, 1=large) and fertilizer (-=0g/plant, +=2.4g/plant) on total stolon length at Miami Whitewater Forest, 17 April 2018

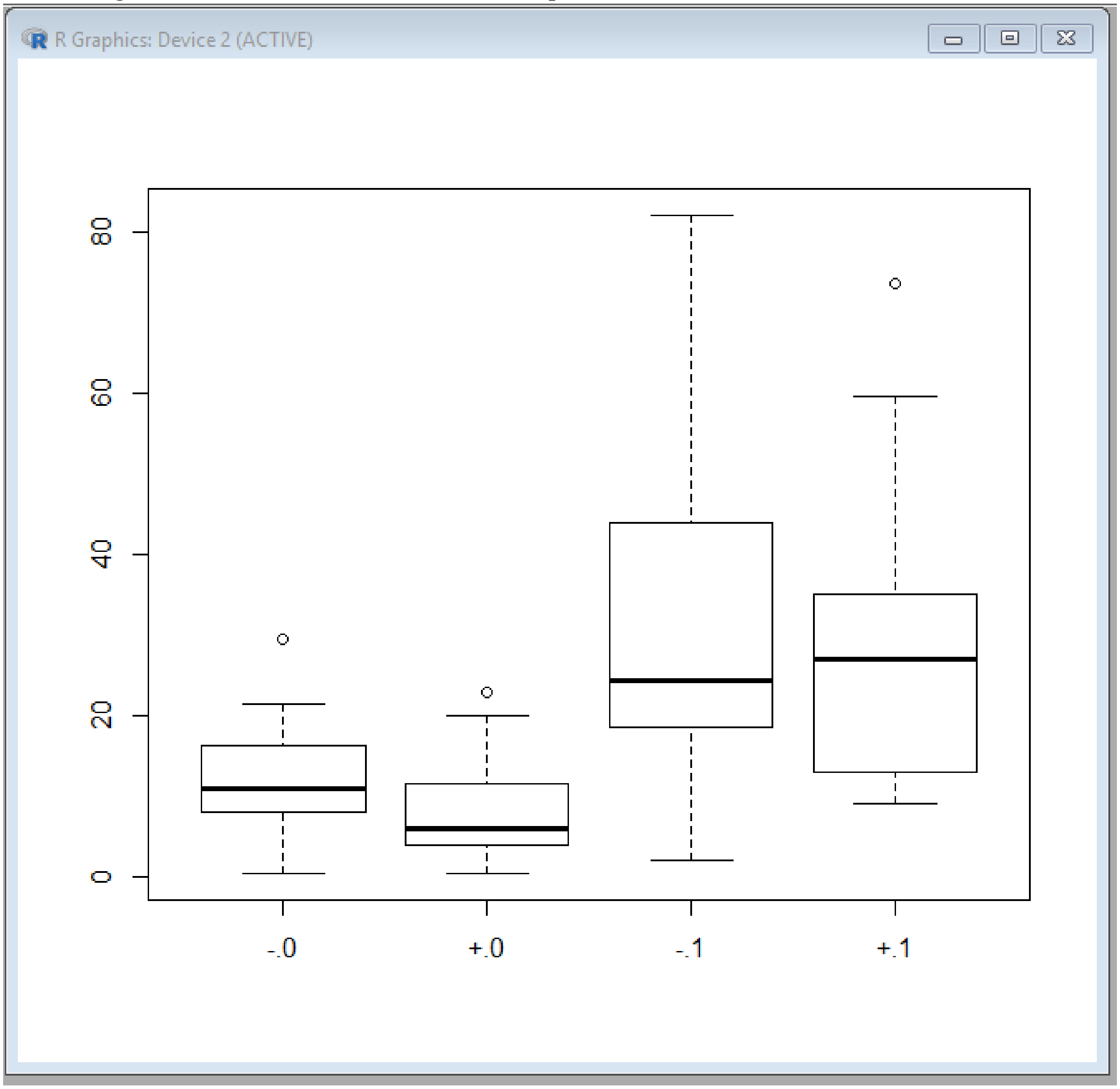


Figure 4.10 Effects of size (0=small, 1=large) and fertilizer (-=0g/plant, +=2.4g/plant) on stolons/plant at Boch Hollow, 17 October 2017

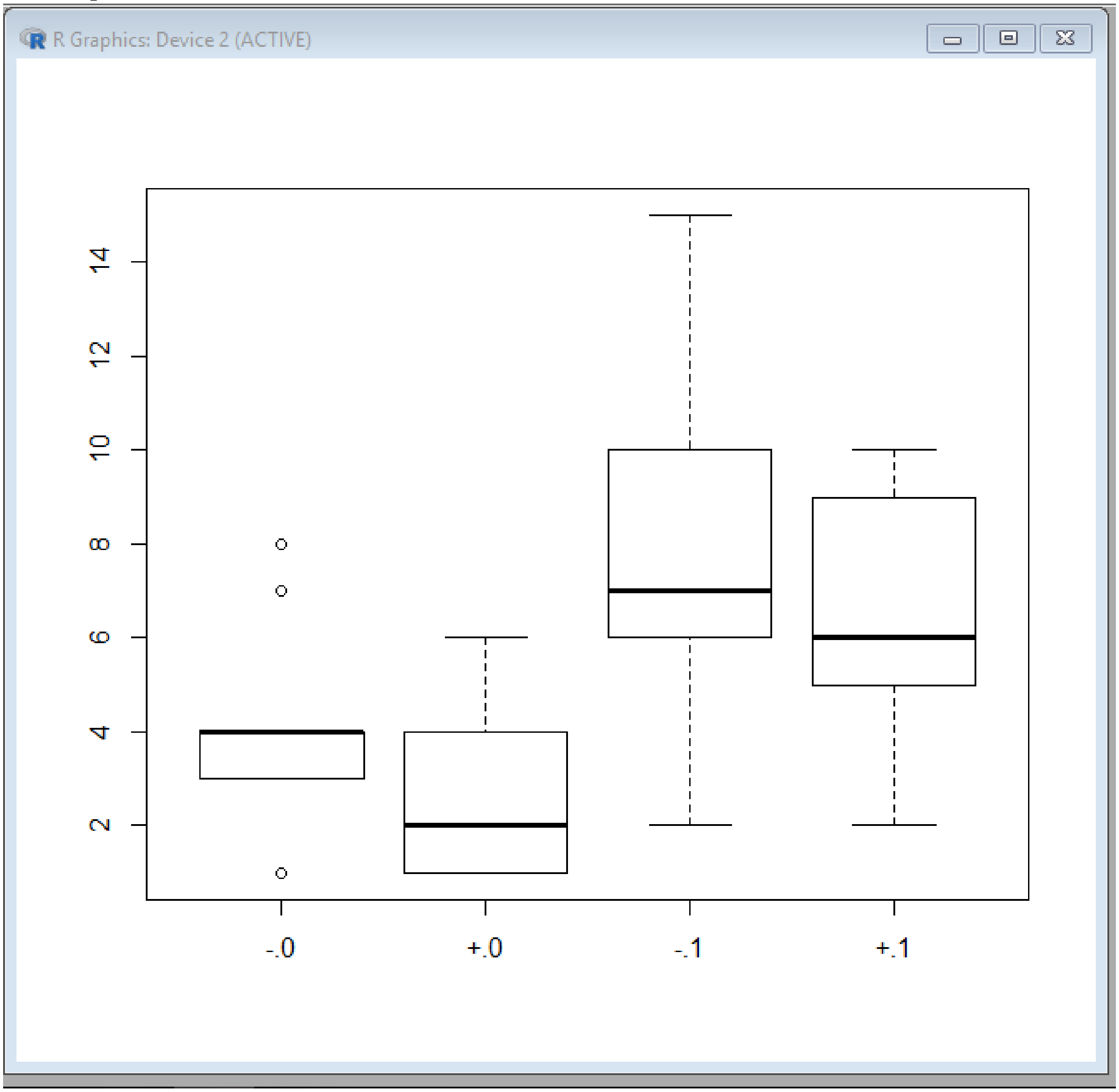


Figure 4.11 Effects of size (0=small, 1=large) and fertilizer (-=0g/plant, +=2.4g/plant) on stolons/plant at Shawnee Lookout, 7 November 2017

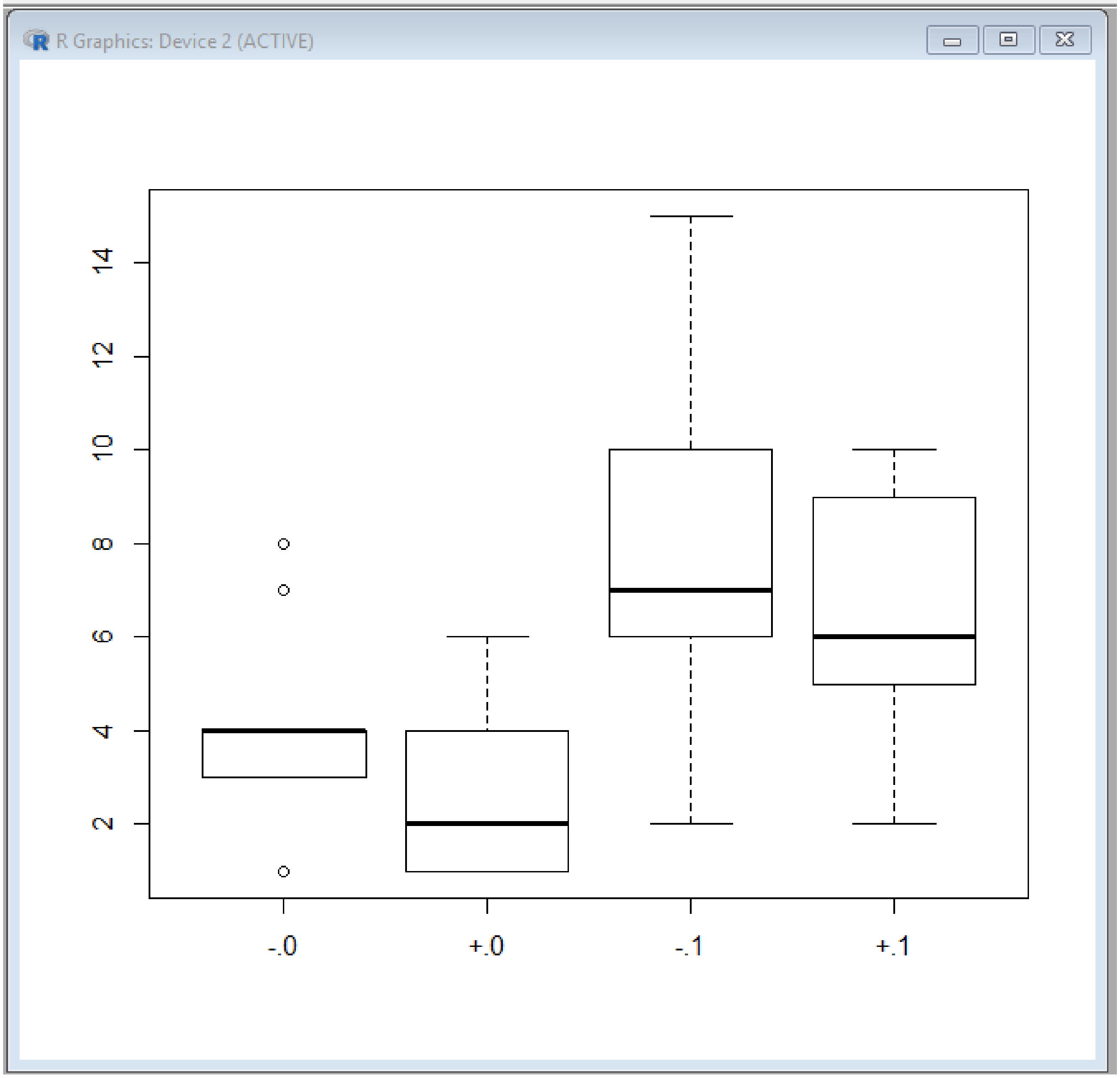


Figure 4.12 Effects of size (0=small, 1=large) and fertilizer (-=0g/plant, +=2.4g/plant) on stolons/plant at Miami Whitewater Forest, 7 November 2017

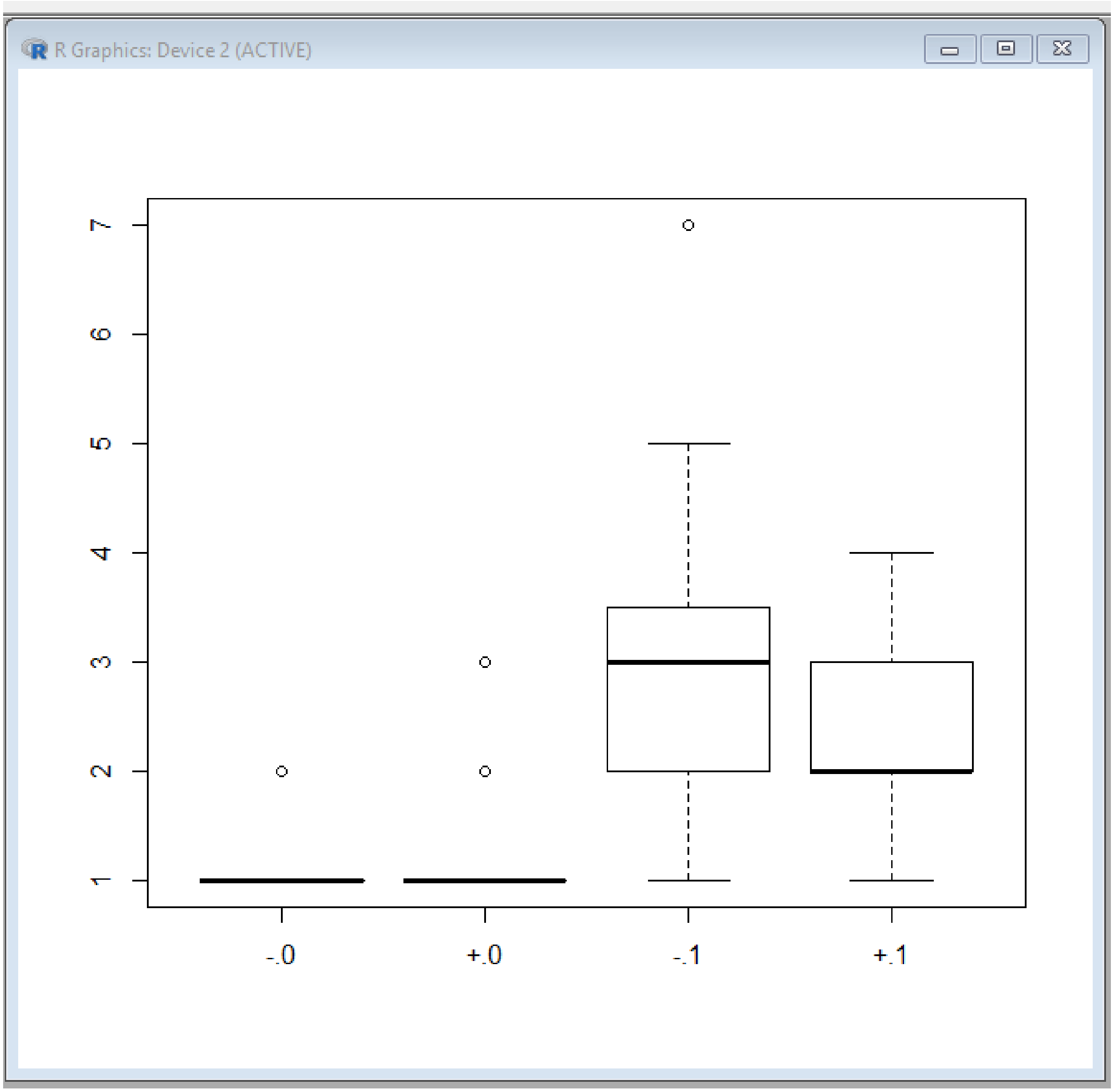


Figure 4.13 Effects of size (0=small, 1=large) and fertilizer (-=0g/plant, +=2.4g/plant) on stolons/plant at Boch Hollow, 10 April 2018

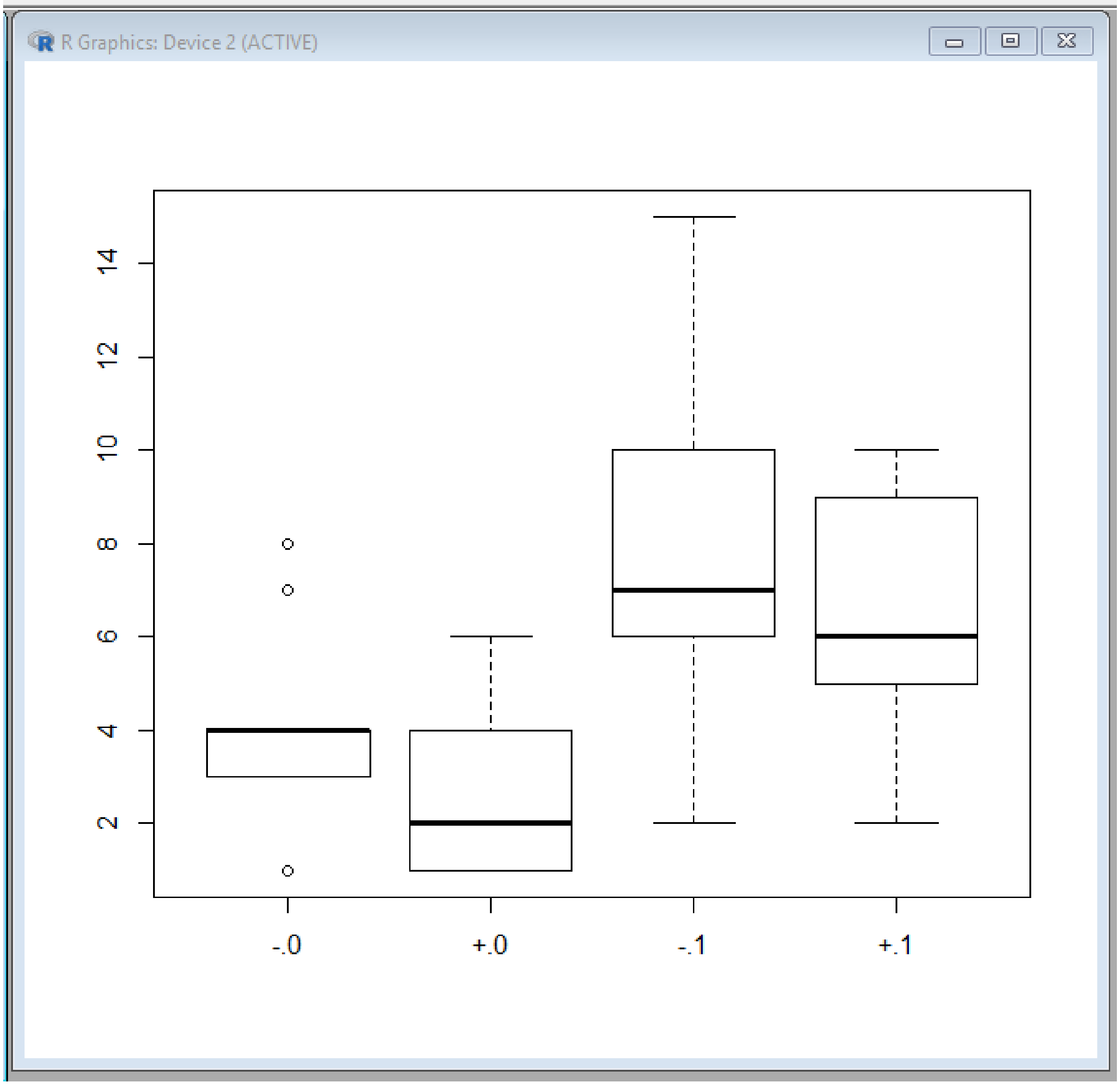


Figure 4.14 Effects of size (0=small, 1=large) and fertilizer (-=0g/plant, +=2.4g/plant) on stolons/plant at Shawnee Lookout, 17 April 2018

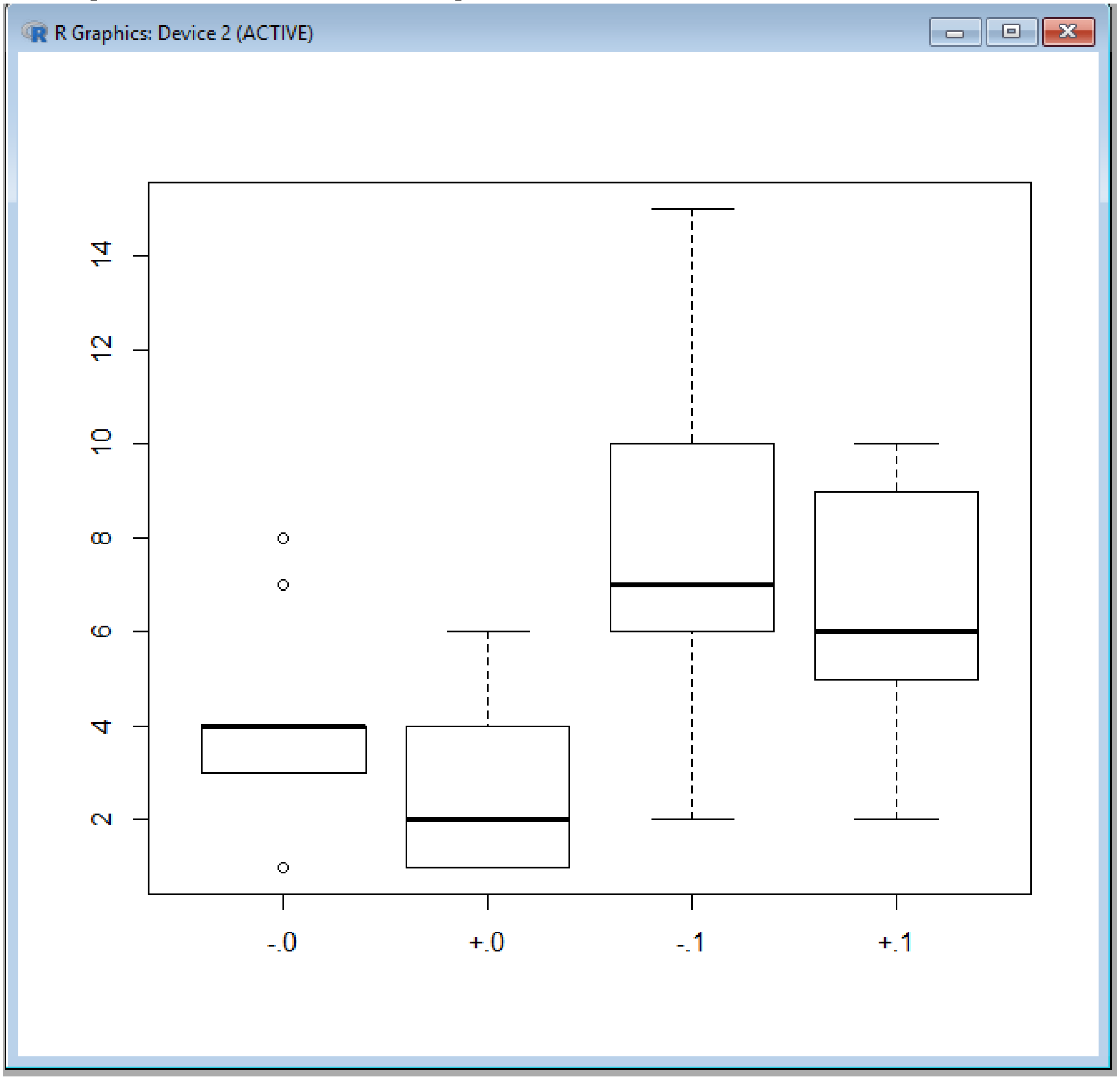
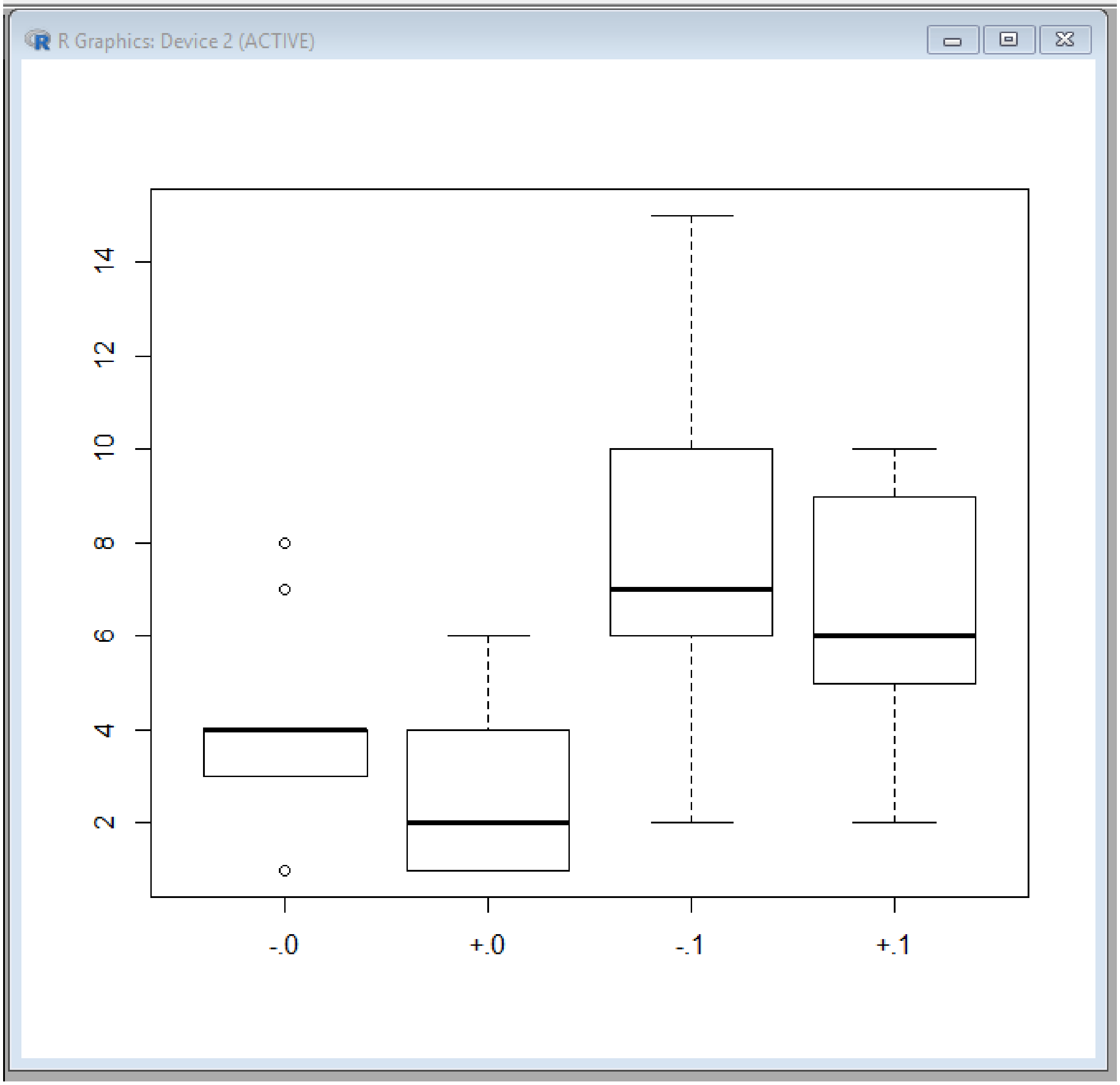


Figure 4.15 Effects of size (0=small, 1=large) and fertilizer (-=0g/plant, +=2.4g/plant) on stolons/plant at Miami Whitewater Forest, 17 April 2018



Chapter 5: General discussion

Running buffalo clover, *Trifolium stoloniferum*, will celebrate 34 years of scientific rediscovery at the time of the publication (Yatskievych 2006). Given the rising awareness of the species within research communities, one might expect that the species, easily propagated in the lab, might have long ago moved from the research greenhouse to vast field trials (Barker and Sparks 2013; Hattenbach 1996). Despite the vast improvements in genetic information and in field monitoring, running buffalo clover still persists in a limited fashion and remains on the Endangered Species List (Crawford et al.. 1998; Hattenbach 1996; Perkins 2015). In the unification of the three proceeding studies, this thesis examined the various ecological, botanical, and biological components of running buffalo clover across three distinct artificial environments: the greenhouse, the laboratory [analyses], and the field plantings. Barring other documentation, the current research program at Ohio State University has explored more facets of the species' biology outside of descriptive in-situ studies. These investigations offered new insights into the species, as well as new observations and direct comparisons between geologically separated populations.

Integrating the results of the above studies into addressing the hypotheses aforementioned in the Introduction:

- Running buffalo clover persists in pH conditions similarly at a regional level for Kentucky Bluegrass accessions, and thus Bluegrass accessions can be treated as a single group of plants
- Phenology and growth strategies is more strongly tied to unique genetics than simply to the population level. Despite genetic similarities (Crawford et al.. 1998), the populations

display moderate morphological diversity, but the degree of diversity is similar amongst populations.

- RBC favors vegetative propagation to sexual reproduction, which would suggest a need to manage plants for stolon production
- Transplants fared well enough to persist through the winter, and outlast previous ODNR/Fish and Wildlife attempts. In surviving this crucial establishment phase, these populations proved that at a minimum, the protocol could work in producing field populations from a fall planting.
- Small transplants without starter fertilizer are capable of establishing over a harsh winter

As mentioned previously, the study failed to investigate the vegetative propagation of wild Missouri, West Virginian, and Kansan RBC material. The Missouri Botanic Garden maintains RBC material from certain sections of the range, as well as historically supported Missouri Department of Conservation reintroductions in the past, but currently doesn't investigate field transplantation and propagation strategies (Smith 1998). Collaboration with institutions, as well as improving access to seed accessions, would improve upon the present task of species recovery. Comparing material performance in the varying ecological microhabitats across the range would improve transplantation protocols, as well as confirm the validity of the protocols developed in the present study. Improving seed access would al.so support propagation ex-situ for research purposes investigating other pertinent questions to the species' biology.

In addressing the technical challenges of transplantation strategy, the author acknowledges that ethical complexities for a species both threatened and maintained by humanity. All sites used in the present study had been disturbed by humans at some point in the last ten thousand years. Boch Hollow's populations were found along a gravel service two-track, Miami Whitewater

Forest’s roughly 200 plants lie along a bike path, and Shawnee Lookout maintains one of the largest populations on one of Ohio’s richest archaeological sites (Fiedel 1992; Scarry 2003; University of Cincinnati 2009). Especially in a region where Indians gathered at high densities and manipulated so much of the environment, it might be fair to suggest that the Indian deserves a rightful place alongside the bison as a clover caretaker (Mills 1914). As much as an ecologist might yearn for the return of bison, so too might they yearn for the Hopewell.

Comparison of successful greenhouse genotypes and field genotypes reveal that for some genotypes, plants perform better in the greenhouse than in the field. In a scheme to maintain field populations ex-situ, this unintentional selection pressure will eventually produce plants less fit for a transplantation scheme (Figures 5.1-3). The “release” from environmental limitations may too play a role; Miami Whitewater Forest plants may not enjoy better growth in the arguably superior conditions of the greenhouse, but Shawnee Lookout genotypes perform especially well away from the natural sites (Figures 5.2-3).

The three studies al.so involved a common collation of previous researchers. Collating observations from three decades and roughly 40 professionals generated several images of the state of running buffalo clover research. Various research programs across the Midwest explored regional populations or facets of the species. Ohio examinations from the 1990s were limited to the Bluegrass populations in Hamilton County as Appalachian populations weren’t discovered for another decade (Hattenbach 1998; Franklin 1996). A decade of field transplantations in Missouri halted when native plants were found by happenstance and interest waned across agencies (Yatskievych 2006). Norman Taylor maintained collections alongside his many other clover species in conjunction with Kentucky ecologist Dr. Julian Campbell (Dr. Julian Campbell

2018, pers. comm). At the regulatory level, ODNR remains focused on protecting habitat rather than on using intervention strategies (Gardner et al. 2017, pers. comm; Selbo et al. 2015).

Within the Kentucky and Missouri strategies of producing plant material, separate historical events led to the discontinuation of active propagation. Norman Taylor's passing led to the University of Kentucky [UK] transferring plant material to the USDA GRIN network; this transfer led to the plant material being maintained passively as accessions rather than actively as a representative of source populations. Even with the transfer of seed, UK lost track of seed accession regeneration, and the transfer of historical collecting information and propagation got lost in the shuffle (Dr. Julian Campbell 2018, pers. comm). After the failure of several Missouri plantings, as well as the discovery of native Missouri plant material, the Missouri Botanical Garden ceased active propagation of plant material and instead collected seed from wild populations, banking said seed for small-scale experimentation (Smith 1998). An unpublished propagation protocol and a published documentation of viral infection exist for the Missouri program, but regular propagation ceased after 1998 (Dr. Matthew Albrecht 2018, pers. comm)).

In this sense Ohio State University's program reinvented in the wheel, developing similar vegetative and reproductive production protocols for field transplanting to Missouri, while maintaining an expansive collections similar to Kentucky. In unifying these approaches, the aforementioned Ohio State studies revived previous conservation avenues as well as created new intervention strategies to preserving running buffalo clover. Through the comparison to agricultural legumes, the edaphic study discovered the uniform responses of Kentucky Bluegrass accessions under varying pH regimes. In actively comparing accessions in a common garden, the morphological characterization study identified phonological and growth differences between populations not readily observed either in the field or under genetic testing. Through the development of improved transplantation protocols, challenges in producing and establishing populations were noted, while al.so applying agronomy to such efforts. The Ohio State strategy established in this thesis took advantage of these existing botanical-ecological conservation strategies and with applied agronomy improved on these previous approaches to conservation by developing parallel strategies and protocols. While the cyclical appearance and disappearance of previous research initiatives mimics the tenuous nature of the clover, the limited communication and information regarding these previous efforts slowed present progress. In producing this thesis, the author hopes to present a summary of past research contacts to future researchers, and expand interest in the species in all three spheres of research: lab, greenhouse, and field.

Figure 5.1. Boch Hollow

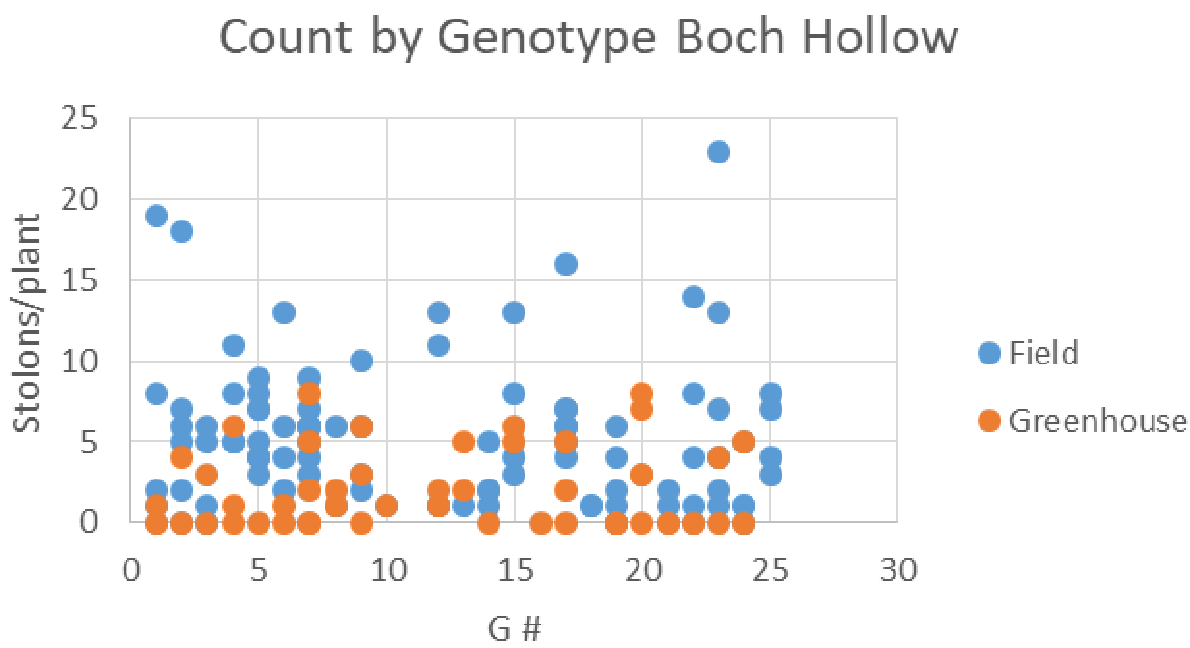


Figure 5.2. Shawnee Lookout

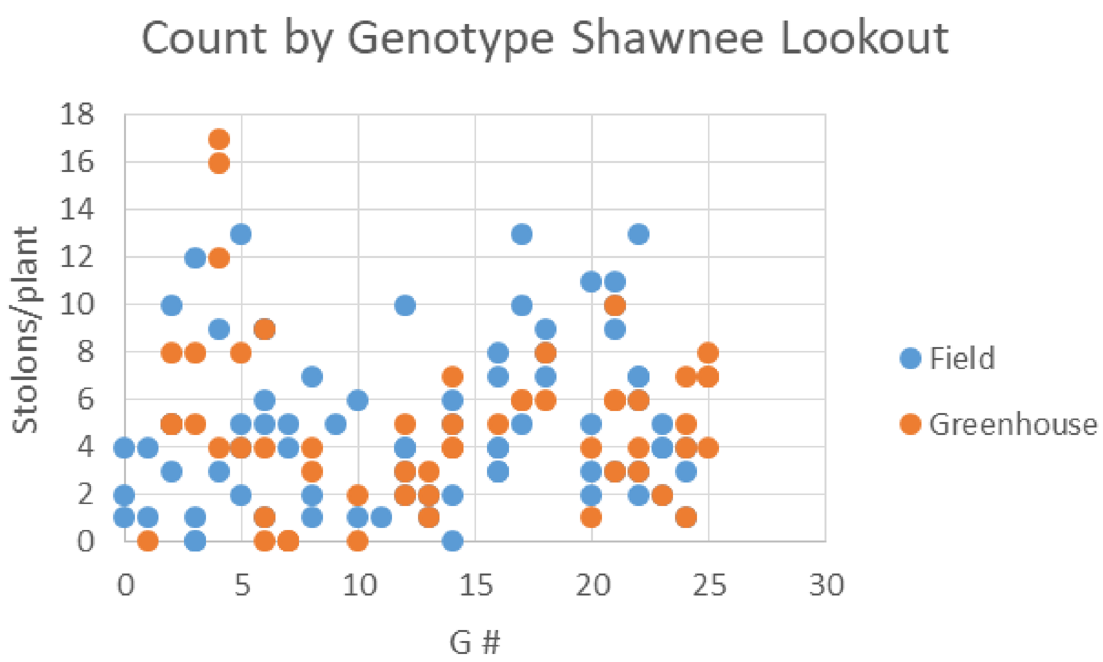
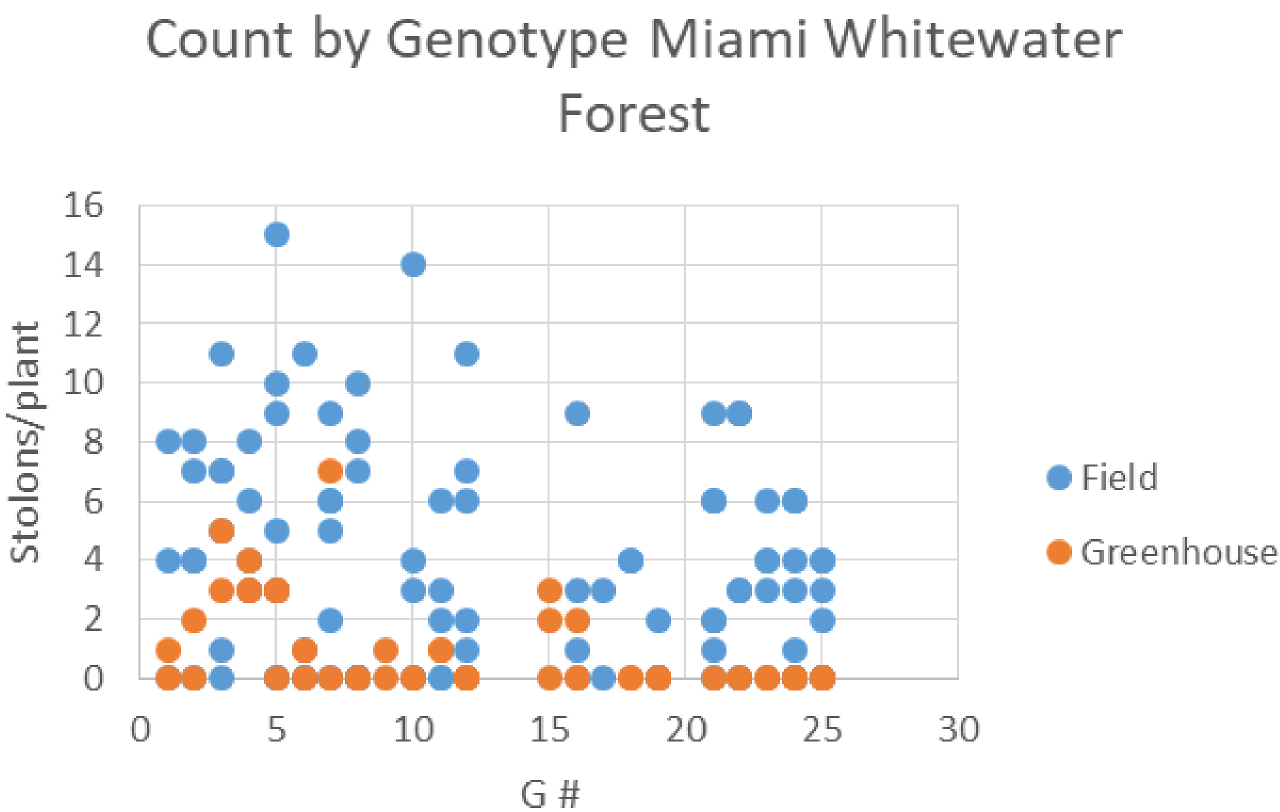


Figure 5.3. Miami Whitewater Forest



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Appendix 1: Chapter 2 R analysis code

Comparison across all Spp/Accession

```
z = read.table('Final pH measures.txt', header=TRUE)

z

aov.out <- aov(pH ~ Spp + Trt + Spp:Trt + Rep, data=z)

summary(aov.out)

aov.out <- aov(pH ~ Trt+ soil + trt:soil + Rep, data=z)

summary(aov.out)

install.packages("agricolae")

library(agricolae)

out <- LSD.test(aov.out,"Spp", main=" Final pH measures ")

out

out <- LSD.test(aov.out,"Trt", main=" Final pH measures ")

out
```

Comparison across Spp

```
y = read.table('Final pH measures SPP.txt', header=TRUE)

y

aov.out <- aov(pH ~ Spp + Trt + Spp:Trt + Rep, data=y)

summary(aov.out)

out <- LSD.test(aov.out,"Spp", main=" Final pH measures SPP ")

out2 <- LSD.test(aov.out,"Trt", main=" Final pH measures SPP ")
```

Comparison within RBC

```
u = read.table('Final pH measures RBC.txt', header=TRUE)

u

aov.out <- aov(pH ~ Spp + Trt + Spp:Trt + Rep, data=u)

summary(aov.out)

out2 <- LSD.test(aov.out,"Trt", main=" Final pH measures RBC ")

out2
```

Appendix 2: Chapter 3 R analysis code

```
morph = read.table("data.txt", header=TRUE)  #input data into the data.frame ‘spotdata’

morph$Accession <- as.factor(morph$Accession)

morph$Genotype <- as.factor(morph$Genotype)

anova (lm(Count~Block + Accession/Genotype, data=morph))

morph = read.table("data.txt", header=TRUE)  #input data into the data.frame ‘spotdata’

morph$Accession <- as.factor(morph$Accession)

morph$Genotype <- as.factor(morph$Genotype)

anova (lm(Bud~Block + Accession/Genotype, data=morph))

morph = read.table("data.txt", header=TRUE)  #input data into the data.frame ‘spotdata’

morph$Accession <- as.factor(morph$Accession)

morph$Genotype <- as.factor(morph$Genotype)

anova (lm(Flower~Block + Accession/Genotype, data=morph))

morph = read.table("data.txt", header=TRUE)  #input data into the data.frame ‘spotdata’

morph$Accession <- as.factor(morph$Accession)

morph$Genotype <- as.factor(morph$Genotype)

anova (lm(Canopy~Block + Accession/Genotype, data=morph))

shapiro.test(morph$Count)

shapiro.test(morph$Bud)
```

```
shapiro.test(morph$Flower)

shapiro.test(morph$Canopy)

morph = read.table("data.txt", header=TRUE)  #input data into the data.frame ‘spotdata’

morph$Accession <- as.factor(morph$Accession)

morph$Genotype <- as.factor(morph$Genotype)

kruskal.test(Count ~ Accession, data = morph)

kruskal.test(Bud ~ Accession, data = morph)

kruskal.test(Flower ~ Accession, data = morph)

kruskal.test(Canopy ~ Accession, data = morph)

#input data into the data.frame ‘morph’

morph = read.table("data.txt", header=TRUE)

# Boxplot of Count by Accession

boxplot(Count~Accession, data=morph)

boxplot(Bud~Accession, data=morph)

boxplot(Flower~Accession, data=morph)

boxplot(Canopy~Accession, data=morph)
```

Appendix 3: Chapter 4 R analysis code

```
install.packages("lsmeans")

install.packages("lme4")

library(lsmeans)

library(lme4)

mydata = read.table("transplant test.txt", header=TRUE)

mydata

mydata$trt<- as.factor(mydata$trt)

mydata$genotype<- as.factor(mydata$genotype)

(fit <- lmer(count~ fert*size + (1 | genotype), data=mydata))

or

lmer(count ~ fert*size + (1 | genotype), data=mydata)

anova(fit) #gives regular anova for fixed effects.

lsmeans(fit, pairwise ~ fert)

mydata = read.table("transplant test.txt", header=TRUE)

mydata

mydata$trt<- as.factor(mydata$trt)

mydata$genotype<- as.factor(mydata$genotype)

(fit <- lmer(length ~ fert*size + (1 | genotype), data=mydata))
```

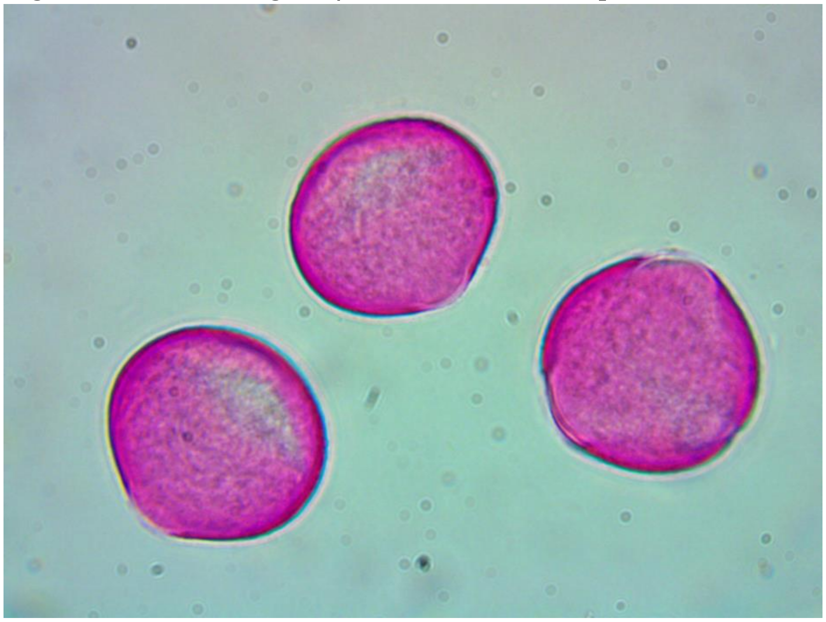
or

```
lmer(length ~ fert*size + (1 | genotype), data=mydata)

anova(fit) #gives regular anova for fixed effects.

lsmeans(fit, pairwise ~ fert)

#input data into the data.frame ‘transplant’

transplant = read.table("transplant test.txt", header=TRUE)

# Boxplot of Count by Accession

boxplot(count~fert*size, data=transplant)

boxplot(length~fert*size, data=transplant), boxplot(length~genotype, data=transplant)
```

Appendix 4: Pollen observations

An initial qualitative examination of running buffalo clover pollen took place March 9, 2018 using a 400X magnification under a light microscope. A source mature flower from a Shawnee Lookout genotype was excised from a three-day-old inflorescence and dissected. The inner stamen and carpel were prepared on a wet mount slide. Anthers and stigma were both scrutinized for pollen. After developing this process, flowers were taken to the Rothenbuhler Honey Bee Research Laboratory for imaging of pollen tubes and appearance March 27, 2018. Representatives from as many populations as possible were processed (Columbus, OH).

Figure 1. Pollen images of Shawnee Lookout specimens



Appendix 5. Professional Contacts

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